THE EFFECT OF AGITATION ON THE CONTROL OF SECONDARY METABOLISM IN LIQUID CULTURES OF *PENICILLIUM EXPANSUM*

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ABSTRACT

The effect of agitation on secondary metabolism in *Penicillium expansum* has been investigated by studying primary metabolic enzyme activities, mycelial lipid accumulation, and the production of the polyketides patulin and citrinin. The results presented here clearly show that agitation directed the flux of acetyl CoA into lipid accumulation rather than polyketide biosynthesis, particularly in the malt extract medium used in these studies. This would be a result of citrate accumulation inducing fatty acid synthesis through the increased activity of the Tricarboxylic Acid (TCA) cycle through increased O_2 concentrations. High glucose concentrations in the Yeast Extract Glucose Buffer (YEGB) medium would result in catabolite repression of this activity, and O_2 diffusion to the mycelium would have been reduced through the thick mycelial suspensions that formed in the shake cultures.

The supply of acetyl CoA to polyketide synthesis, controlled through the availability of O_2 to respiratory processes, is considered a major regulatory factor in this organism. The availability of reducing power in NADPH was also a significant factor favouring lipid synthesis over polyketides in shake cultures. The pentose phosphate pathway was more active in shake cultures than static, as were the other potential sources of NADPH in the cytoplasm - malic enzyme and the $NADP^+$-utilising form of isocitrate dehydrogenase.

The ammonium concentration was not likely to repress polyketide synthesis in either medium. There was some evidence to suggest that patulin synthesis was affected by feedback inhibition of late pathway metabolites or by intracellular protease activity.

Finally, the presence of O_2 also affected the route taken by intermediates of patulin biosynthesis. The presence of toluquinol in the shake culture filtrates was possibly due to O_2 activation of a mixed function oxidase competing for *m*-cresol with another producing *m*-hydroxybenzyl alcohol, normally present in static cultures.

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PREFACE

This thesis is based upon the work of Woodhead and Walker (1975), who originally showed that the aeration of liquid cultures of *Penicillium expansum* negatively affected the production of patulin. That paper formed the foundation of these studies, which sought to build upon and extend the knowledge already gained.

The approach taken in this thesis was to split metabolism into different areas in order to build a picture of what happens when cultures of *P. expansum* are agitated. It was important to ascertain whether or not the trigger for patulin production to occur preferentially in static cultures was a result of a direct effect on secondary metabolism or if it was a flow-on effect from primary metabolism.

Enzymes representing the major metabolic pathways in primary metabolism were assayed, with the exception of the electron transport chain, with a view to those in particular that might be involved in NADPH generation, since this is an important molecule in biosynthesis. The generation of NADPH can be assumed to occur under conditions which favour primary processes, and would therefore be indicative of primary metabolism being favoured.

The mycotoxin patulin was the focus of the work in this thesis. This polyketide is a model compound for other secondary processes because its biosynthetic pathway has been established, as well as mechanisms for its regulation, such as ammonium repression and termination of synthesis by intracellular proteinase activity. A comprehensive understanding of the mechanisms surrounding the synthesis of this compound is beneficial through the application of this knowledge to other polyketides that are important industrially, such as antibiotics. This can then allow the manipulation of fermentations to achieve better yields of the chemical of interest, by metabolic engineering, or the removal of physiological constraints.

Therefore the primary objective of this study was to determine how aeration through agitation affects secondary metabolism in *P. expansum*, by exploring the effects on primary pathways, lipid accumulation and extracellular enzyme activities.

CHAPTER 1

OXYGEN

1.1 OXYGEN AND MYCELIAL GROWTH

Oxygen is a colourless, odourless, gaseous element which makes up 28% of the earth's atmosphere by volume, and which is vital for the survival of aerobic organisms due to its function in respiration. The respiratory enzymes of microbial cells are in an aqueous environment, so cells are only able to utilise dissolved O_2 (DO). At 20°C and in an atmosphere of air, water only holds 9 parts per million (ppm) O_2, this amount decreasing with increasing temperature. The solubility of oxygen is largely independent of the presence and pressure of other gases (Finn, 1954).

Cell respiration will occur at a rate independent of the DO concentration, provided this concentration exceeds a threshold level (ie. the critical oxygen tension), below which the oxygen uptake rate resembles Michaelis-Menten kinetics and which correlates to the high O_2 affinity of the terminal oxidase of the respiratory electron transport chain (Harrison, 1972). For unicellular organisms this critical level is low, and the respiration rate is limited more by the unsaturation of enzyme surfaces than slow diffusion of O_2 into the protoplasm. The respiration rate of multicellular organisms is more likely to be affected by the rate of diffusion of oxygen, and the rate of supply of the substrate will dictate the rate of respiration. The demand for oxygen by a submerged microbial culture is dependent on various factors, including the concentration and form of nutrients in the medium, the accumulation of toxic end products, the loss of volatile intermediates, and the supply of O_2 to the medium (Finn, 1954).

The Ionic strength of media and aqueous solutions has been shown to affect the transfer and behaviour of O_2, increasing ionic strength resulting in decreased O_2 solubility (Hansberg and Aguirre,1990). Robinson and Wilke (1973) showed variations in ionic strength affected

properties such as viscosity, density and diffusion in a basal salts medium, and were able to predict the volumetric coefficient (k_La) for mass transfer from dispersed gas bubbles to the fermentation medium. The volumetric mass transfer coefficient is the main parameter describing the performance of a bioreactor, since liquid-phase resistance controls the overall O_2 transfer rate (Kawase and Moo-Young, 1988). The dispersion of air bubbles in aqueous electrolyte solutions was studied by Zieminski and Whittemore (1971). The bubbles were shown to decrease in the degree of coalescence depending on the concentration of salts, so that the rate of O_2 transfer was increased considerably with increasing salt concentration. Anionic surface-active agents were shown to have an adverse effect on the rate of solution of O_2 in aerated water (Downing *et al.*, 1957).

Several resistances are encountered as O_2 is transferred from the gas phase to the cellular site of utilisation. The main resistance is in the form of stagnant films μm to mm in thickness, which are dependent on the degree of turbulence and the physical properties of the medium, and which occur between the bulk of the gas and the gas-liquid interface, and between the gas-liquid interface and the bulk of the liquid. There are liquid films around individual cells or cell clumps, as well as intracellular and intra clump resistances (Finn, 1954). Resistances are also provided in the form of the area of gas-liquid contact, the time of contact, and the intensity of agitation of the culture medium.

Whether or not a cell grows singly or in a clump (a mycelial aggregate) can be a factor in the resistance encountered in the surrounding liquid films. For cell clumps, liquid films are more important since there is more likely to be relative motion between a clump and its surrounding fluid, and the intra-clump resistance is considerably greater than that of the liquid film. Anaerobic conditions occur within a clump once a certain size is reached and O_2 uptake into a clump will determine its size. However it must be noted that shear can contribute to the breakdown of cell clumps.

Olsvik *et al.* (1993) studied clumps in *Aspergillus niger* fermentations by image analysis and showed that 89-99% of fungal material was growing in clumps despite the visual observation that there was a fully filamentous growth form (this level of clump growth was predicted to be common in mycelial liquid cultures of filamentous fungi). The consistency and size of clumps were believed to be related to its roughness and compactness at constant biomass

concentrations. Roughness correlated to specific growth rate of the culture, and increased with increasing O_2 tension. The O_2 was possibly having an effect on hyphal-hyphal interactions within the clumps or on the rigidity of cell walls, and not involved in clump breakdown. Zetelaki and Vas (1968) suggested that DO tension may affect cell wall composition and therefore the flexibility of the hyphae. Oxygenated cultures (i.e. oxygen introduced to the culture) of *A. niger* had less well-defined and less rigid cell walls than aerated cultures (i.e. air introduced rather than oxygen gas), therefore making them resistant to mechanical agitation, resulting in lower viscosity.

Several authors have been concerned with the effect microbes have on the O_2 k_LA. Wise *et al.* (1969) showed that in medium aerated at a low Reynolds number flow (i.e. laminar flow-type conditions; -the Reynolds number is a dimensionless indicator of turbulence within the system, and is a means for describing the flow regime which exists in the system). According to Banks (1977), the rate of O_2 transfer was increased due to the presence of microbial cells at the liquid surface film. The physical properties of the medium were not changed by the fermentation, instead it was proposed that the effect was due to the disruption of the liquid surface film around the O_2 bubbles. The organisms used were *Candida intermedia* and *Pseudomonas ovalis*, and other studies have shown similar results with unicellular organisms such as *Escherichia coli* (Andrews *et al.*, 1984). King and Palmer (1989) concluded that because increased O_2 transfer was observed with *Bacillus licheniformis* and not *Micrococcus luteus* that the accumulation effect was related to motility. Ju and Sundararajan (1995) showed the overall effect of *Saccharomyces cerevisiae* cells on O_2 uptake was positive, and was not due to their presence in the layer near the bubble surface (which was actually concluded to reduce permeability to O_2), but instead due to cell respiration and medium modification, contrary to the findings of Wise *et al.* (1969). Further, the observed effects occurred at high agitation speeds and low aeration rates and were attributed to the interfacial cell accumulation associated with the smaller bubbles produced under these conditions. The blocking effect of non-respiring cells was also observed in previous work by these authors (Ju and Sundararajan, 1994), and Huang and Bungay (1981) showed the presence of a stagnant 'skin' in static *P. ovalis* cultures which gave resistance to O_2 transfer.

The nature of O_2 uptake has been studied in yeast cultures in which an increased rate of uptake had been observed. Sobotka *et al.* (1981) proposed a two-phase uptake model for aerobic

fermentations where O_2 uptake can occur from both the liquid and gaseous phases. A model was derived for two-phase uptake and provided a better fit for the experimental results than if liquid phase only was being considered.

During the course of penicillin-producing fermentations of *Streptomyces griseus* the O_2 uptake rate changed through the life cycle of the organism and the accompanying changes in fermentation conditions (Bartholomew *et al.*, 1950). Ju *et al.* (1991) showed that changes in CO_2 composition in the gas supply to *Penicillium chrysogenum* fermentations had a dramatic effect on broth rheology and k_La, since this fungus varied in form from normal mycelia to pellets. This could possibly find a use in controlling culture morphology and therefore O_2 transfer capabilities.

1.1.1 Agitation and aeration.

Improvements in the interfacial area between gas and liquid can be brought about by agitation. The airstream is chopped into smaller bubbles (and therefore the area available for O_2 transfer increases), and swift eddies in the circulating media prevent escape and coalescence of bubbles. The turbulent shear created also reduces the thickness of the liquid film (Banks, 1977). Beyond agitation, resistances can be overcome by increased partial pressure of O_2 (pO_2) using O_2-enriched air, or air under pressure, however the latter has the undesirable consequence of the sweeping effect of nitrogen (i.e. given that the relative volume concentration of nitrogen in air is greater than oxygen, the nitrogen will "blanket" the culture, excluding oxygen), and an increase in CO_2 solubility. Inadequate CO_2 becomes a problem when pure O_2 atmosphere is used (Finn, 1954).

Unicellular microorganisms, such as yeast and bacteria, in liquid culture are said to behave as Newtonian fluids, which means they follow Newton's law of viscous flow. The velocity gradient in the fluids is directly proportional to the shear stress. When a force is applied to a liquid it will normally flow, and the viscosity of the liquid may be looked upon as its inherent resistance to flow (Banks, 1977). Single cells have a liquid film at the cell wall, which offers no resistance to the diffusion of O_2. The thickness of this film can only be reduced by increasing the relative velocity between the cell and the fluid, however the cells follow fluid streamlines since their specific activity in the culture medium is not sufficient enough to

overcome the inertia due to their small size. In this case increased growth rate is due to agitation side effects such as relief of saturation of CO_2 in the culture medium produced by the organism (Finn, 1954).

For fungi the effect is completely different. Fungal mycelia are considerably larger than unicells and greater in size than the eddies in which turbulent energy in fermentors is dissipated, therefore dampening the turbulence and consequently the k_La (Andrews *et al.*, 1984). Mycelial cultures behave as non-Newtonian fluids i.e. they do not possess a true viscosity, only an 'apparent' viscosity, which is not a constant, characteristic, property of the fluid and which is dependent on the shear rate. Banks (1977) describes four non-Newtonian fluids of interest to fermentation workers:

(1) Bingham plastics: similar to a Newtonian fluid but different because there is a limiting shear stress which must be exceeded if a liquid flow is to occur. At rest these fluids have a three-dimensional structure of sufficient rigidity to withstand any stress less than the yield stress, however once the yield stress is exceeded the fluid behaves in a Newtonian manner.

(2) Pseudoplastics: the apparent viscosity decreases with shear rate, and example of which is polymer solutions (which could be analogous to hyphal solutions) where at progressively higher shear rates the long chain molecules of the polymer tend to align with each other and thus slip more readily, causing decreased viscosity. Cultures of *Streptomyces niveus* were O_2-limited despite a 70% DO saturation since the viscosity of a pseudoplastic liquid increases with increasing shear stress, resulting in broth viscosity increasing with distance from the impeller in a fermentor (Bushell, 1989).

(3) Dilatants: the apparent viscosity increases with increasing shear rate.

(4) Casson body: these possess a well-defined yield stress whilst the apparent viscosity decreases with increasing shear rate ie. combining the properties of a Bingham plastic and a pseudoplastic. This occurs in many concentrated suspensions.

Mycelial suspensions exhibit non-Newtonian properties because the branched mycelial network forms a three-dimensional structure, which gives the suspension rigidity, and therefore results in a yield stress. The long hyphae tend to align with each other with increasing shear rate, appearing pseudoplastic in nature. Most published work indicates that cultures of hyphal organisms behave either as pseudoplastics, Bingham plastics or Casson

bodies (Banks, 1977). The behaviour of the culture can change according to the age or stage of the fermentation, a *Streptomyces griseus* strain culture fluid behaved as either Newtonian or Bingham plastic depending on age; Bingham plastic occurred approximately midway through the fermentation, and Newtonian behaviour was displayed during the initial and final stages of the fermentation. The final period of the culture was Newtonian due to increased lysis and fragmentation of the mycelium (Banks, 1977). During the course of any fermentation the cell density will increase and the morphological characteristics of the organism such as degree of mycelial branching will alter and therefore have an effect on the culture rheology. In the initial stages of the fermentation the organism will be distributed in the medium as small discrete mycelial agglomerates, in which case the culture fluid will behave as a Newtonian fluid. Later, once the three-dimensional hyphal structure has formed, non-Newtonian behaviour will result and finally, as mentioned earlier, partial or complete lysis will occur and the culture would be expected to revert to Newtonian behaviour (Banks, 1977).

There are a number of difficulties involved in cultures that exhibit non-Newtonian fluid behaviour according to Banks (1977). The first is that the high viscosity of many fungal and streptomycete cultures prevents conditions of fully developed turbulent flow from being established, so that within such cultures the flow conditions in the fermentor lie in a transition region between the regions of viscous and turbulent flow. In the transition region the relationship between operating variables and power consumption are complex, and little is known about the relationship between power consumption and aeration efficiency. Secondly, non-Newtonian fluids do not possess a true viscosity, only an apparent viscosity which is not constant and dependent upon shear rate. Since shear rates vary within the fermentor, the apparent viscosity will therefore also be variable, and a complex system will therefore exist which will defy theoretical analysis. Thirdly, there is a lack of information available concerning the rheological behaviour of filamentous microorganisms.

Under normal operating conditions the O_2 efficiency of a culture seldom exceeds 2% and is often below 1%. Shake flasks are adequate on a small scale because O_2 transfer takes place by a diffusion process through the surface liquid film which is constantly renewed under the conditions (Banks, 1977). Shaking is also considered better than bubbling devices since there is a shallow layer of culture medium, however a major drawback of this method is the

production of head foam (Finn, 1954). Shaking is impractical on the large scale because the liquid surface area/volume ratio decreases with increasing scale, the best form of aeration in this situation is provided by stirred fermentors (Banks, 1977).

Two forms of shaking devices have been utilised in aeration of submerged cultures-the reciprocating shaker and the rotary shaker. Reciprocating shakers are less-favoured than rotary ones; a major problem being inconsistent fluid surges in which a wave-like curl return causes unpredictable and uncontrollable 'geyser'-like splashes. There are often substantial viscosity effects, and evaporation of the medium frequently affects the motion of the fluid being shaken. The rotary shaker creates a constant swirl pattern which is less affected by media viscosity, machine start-up or flask volume than the reciprocating shaker. It is also capable of holding heavy and non-uniform loads.

Freedman (1970) determined the extent of sodium sulphite oxidation in shake flasks to calculate O_2 absorption rates (OARs). This work showed the OAR to increase with increased shaker speeds (best performances were above 300 rpm), increased temperature, and when lower flask volumes were used (eg. medium volume 10% of flask volume). Smaller flasks (10 and 25 mL) gave the best OAR results and interestingly the widely-used 250 mL Erlenmeyer flasks gave the poorest results. The size of the stroke of the rotation was also important-the larger the stroke, the better the OAR. Baffled flasks gave better OAR values, but resulted in less uniform cultures.

A system using sparged aeration with shaken flasks was described by Donovan *et al.* (1995), in which the advantages of the shake flask method were retained, but the overall performance improved with a sparging apparatus added to aerate the broth during shaking. The authors claimed a doubling of growth rate of *E. coli* cells by 8 hours in the sparged system over the non-sparged cultures.

A different means of aeration efficiency was demonstrated by Gbewonyo and Wang (1983), who studied penicillin production by *Penicillium chrysogenum* grown in a 3 L bubble column fermentor. The fungus was allowed to grow either as free cells, or confined to Celite beads, and the results proved that the restructuring of the filamentous morphology of the cells resulted in significantly higher k_La values, growth rate and penicillin production than for the

free cell cultures. The specific energy consumption of the process was almost halved in the Celite bead cultures, and required less input aeration. These results showed that free mycelial growth caused a decline in the mass transfer capacity attributed to the high non-Newtonian viscosities of the fermentation medium that resulted.

Over-agitation, or over-aeration, is one problem that has been encountered. The altered yields attributed to this have been difficult to interpret since other factors such as evaporative cooling of the culture, pH changes, acceleration of the degradative reactions, foaming and the use of anti-foaming agents, and the loss of volatile intermediates such as acetaldehyde or CO_2, can all have an effect (Finn, 1954). Mycelial age is important where over agitation is concerned. Older, more fragile hyphae may be prone to greater lysis under high speeds, whereas younger mycelia tend to be more robust and unlikely to rupture (unless abrasive particles are present).

1.1.2 Oxygen and Pelleting.

Under certain conditions submerged fungal cultures grow in the form of pellets ie. spherical compact masses of hyphae. Pelleting has been attributed to low concentrations of conidial inocula (Camici *et al.*, 1952), for example 2-3 x 10^5 conidia/mL for *Penicillium chrysogenum* (Trinci, 1970). It is thought to be dependent on certain fungal strains, and nutrient media and low agitation have been shown to have an effect (Phillips, 1966). Olsvik *et al.* (1993) observed small pellets (less than 5 mm) of *A. niger* occurring at 40 % DO in the broth, and at a low growth/dilution rate in a fungus which tended to grow in clumps otherwise.

Many fungi form pellets when grown in shaken submerged cultures, probably due to an aggregation process which may occur between small clumps and through entanglement of spores in hyphae (Trinci, 1970). Nielsen (1996) refers to three groups of pellet-forming microorganisms according to their mechanism of pellet development: (1) The spore coagulating type, where germination of coagulated spores results in a pellet being formed (eg. many *Aspergillus* species); (2) The non-coagulating type, where a single spore forms a pellet (eg. some *Streptomyces* species); and (3) The hyphal element agglomerating type, where hyphal elements agglomerate and form a clump of hyphal elements which eventually form a pellet (eg. *Penicillium chrysogenum*).

Pelleting maintains low viscosities and high aeration efficiencies, although the nature of pellet morphology prevents effective utilisation of the available O_2. Phillips (1966) showed with *P. chrysogenum* the larger the pellet, the lower the critical O_2 tension in the interior, but the greater the DO of the broth, and vice versa.

Trinci (1970) proposed zones relating to growth efficiencies within *Aspergillus nidulans* pellets, and this is illustrated by Figure 1, and explained these by using cube root growth kinetics for fungal pellets proposed by Pirt (1966). Cube root growth kinetics is a feature of pellet growth, which is affected by the texture/density of the pellets concerned. Pellets with densely-packed hyphae would allow simple diffusion only of nutrients and O_2 to the interior, yet pellets with a looser texture could be supplied with nutrients and O_2 by the turbulence of the medium.

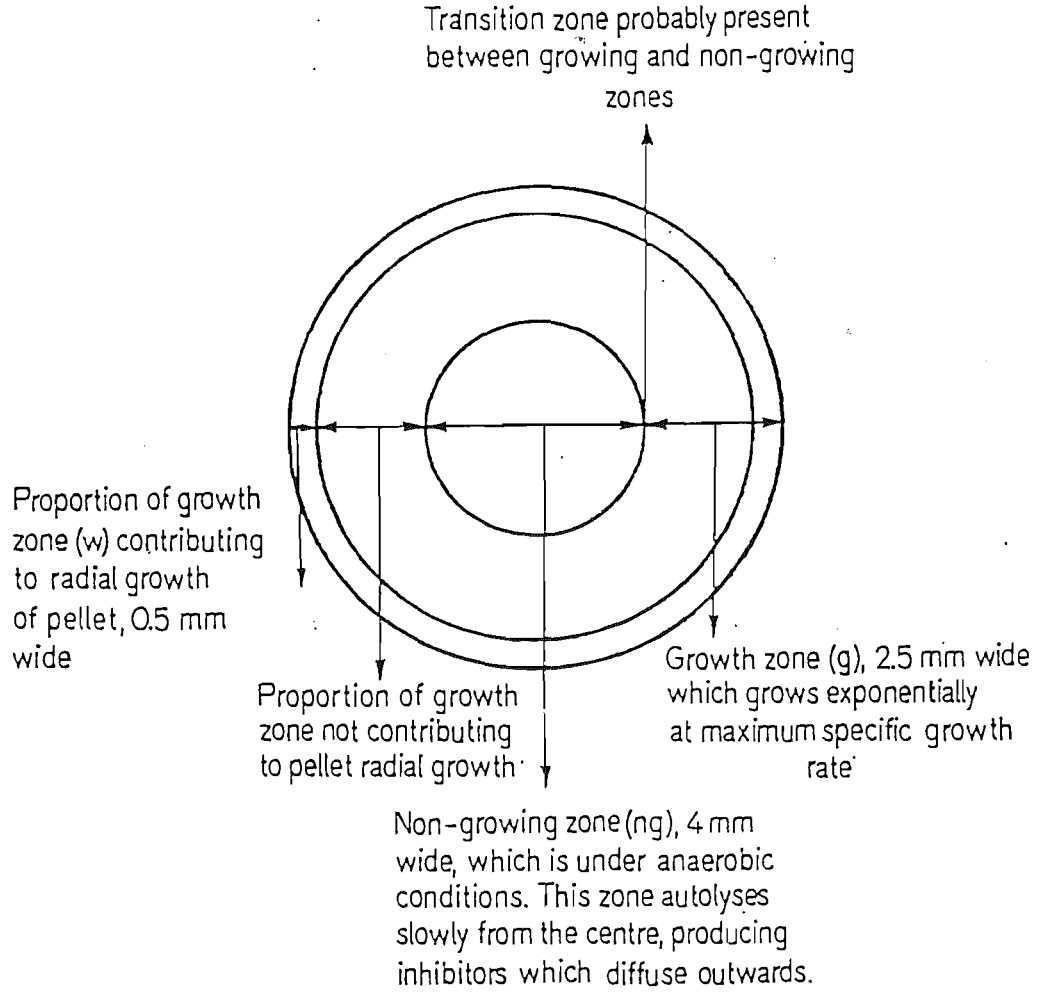


Figure 1. Zones of growth in fungal pellets (from Trinci, 1970).

According to Trinci, an outer growth zone grows exponentially at the maximum specific growth rate with only the outer portion of this region actually contributing to the radial growth of the pellet. Anaerobic conditions are assumed to prevail in the interior, leading to degenerative processes such as autolysis, or production of growth-inhibitory substances, the diffusion of which may contribute to the deceleration of radial growth rate at a specific width for example. Autolysis of pellet interiors leading to an inner space has been reported previously (Camici *et al.*, 1952), and in a *Penicillium urticae* strain, sporulation had occurred at the centre of the pellets in a process analogous to the central region of surface cultures forming arthrospores, where both regions were considered to be deprived of O_2 (Yanagita and Kogane, 1963).

Hansberg and Aguirre (1990) produced a theory to account for differentiation processes in cells that would appear to have some relevance here. The theory states that cells are in a stable state during growth and once they have reached a differentiated state, however the switch from one to the other requires instability and a hyperoxidant state is proposed as the mechanism for this to occur. Hyperoxidant states come about due to the production of excess reactive O species such as $^1O_2$, O_2^-, HO_2, H_2O_2, and $HO\cdot$ which are unable to be neutralised adequately by the cell, or if excess O_2 is able to enter the cell and therefore its metabolism. It is believed that a hyperoxidant state could contribute to processes such as protein carbonylisation through oxidation, which have been shown to coincide with different phases of development. Inherent in this theory is that once a cell reaches a differentiated state, or is part of a differentiated structure, it becomes isolated from the environment and therefore any O_2 that may be present. The differentiation processes that occur inside pellets could be due to this but the internal pellet environments would be expected to be anaerobic so that such a process might not be relevant. Ultimately the pellet interior is isolated from O_2 so that the isolated differentiated cells fit into this theory in this way.

A process which occurs as a result of the non-growing central region of the pellet is pellet break-up which is attributed to the age of the hyphal pellet with respect to the amount of substrate depletion that has occurred at the centre, and to how fast cell lysis has occurred in this region. Medium shear forces also play a role in this process (Nielsen, 1996).

Surface erosion of pellets (pellet erosion) also occurs as a result of culture hydrodynamics, and describes the rupture of hyphae at the pellet surface, resulting in a reduction in the ability to interact with the surrounding medium (Nielsen, 1996).

Huang and Bungay (1973) used a microprobe to determine that the depth at which O_2 was very limiting in *A. niger* pellets was 100 μm, however this work was done on a mounted pellet for which the flow velocity of the medium would have been in excess of that expected by a free pellet in a standard fermentation, and therefore provide a greater O_2 supply than would be expected. Increased pellet sizes result in increases in specific respiration rate of *A. niger* pellets and in the apparent K_m for O_2 for respiration, leading to the conclusion that mycelia in the centre of the pellets were adapted to the low O_2 concentration, therefore discounting the possibility that there might have been uniform respiration through the pellet, or that respiratory capacity was affected by age (Kobayashi *et al.*, 1973).

1.1.3 Functions of O_2 in microorganisms.

O_2 performs a major role in aerobic organisms as the terminal electron acceptor in the electron transport chain. It is utilised in a sequential mechanism where 2 electrons (transferred from the Krebs/TCA cycle as NADH + H^+) are transferred from cytochrome a_3 of cytochrome oxidase to form O^{2-}, which associates with 2 H^+ from $FADH_2$ (produced in succinate dehydrogenase and $NADH_2$ reductase reactions) to form H_2O. Cytochromes play an important role in respiratory metabolism and the levels of these have been shown to adapt in response to varied O_2 tensions. Harrison (1972) reviewed several bacterial (including obligate aerobes and facultative organisms) and yeast cytochrome responses to various DO concentrations and concluded that, with the exception of one culture, a maximum cytochrome content was present in cells under O_2-limited conditions. Facultatively anaerobic bacteria also contain cytochromes which can be killed by O_2, thus indicating the importance of the critical O_2 tension (Mukhopdhyay and Ghose, 1976).

O_2 also functions as a nutrient for some microbes. Yeasts and fungi incorporate O_2 into unsaturated fatty acids and sterols which are essential for cell growth, and where anaerobic conditions lead to the inability to form these compounds, it is necessary to add unsaturated fatty acids to the medium. At low O_2 concentrations, this nutritional requirement leads to

competition between synthetic and respiratory pathways. Methane-utilising bacteria also require oxygen and obtain it from medium DO and O-containing compounds. The methane carbon atom is oxidised by oxygenase enzymes (Harrison, 1972). Another example is catechol-2,3-dioxygenases produced by some *Pseudomonas* species, which functioned in hypoxic environments, utilising NO_3^- as an electron acceptor in the absence of O_2. The kinetic parameters of this enzyme reflected the nature of the environment allowing it to function, and it was synthesized in response to limiting O_2 concentrations (Kukor and Olsen, 1996).

The regulation of metabolism in facultative anaerobes is another role for O_2. Medium pO_2/DO levels induce or repress pathway enzymes in these organisms-the inductive effects occur at levels well below the critical level for respiration. Facultative anaerobes continue to follow anaerobic pathways for biosynthetic reactions, yet will use O_2 where possible as a terminal electron acceptor-repressing the synthesis of systems required for anaerobic energy metabolism (Mukhopdhyay and Ghose, 1976).

O_2 can also act as an energy generator in microorganisms and does so in two ways. Firstly, biothermochemical reactions can occur to generate heat, which can be converted into electrical energy. Glucose and O_2 are converted to CO_2 and water, yielding 673 kg cals. The second reaction is the synthesis of ATP through oxidative phosphorylation.

Finally, O_2 can control the rate of active transport in the cell through DO concentrations at the cell surface which in turn controls product synthesis in oxygen-requiring pathways as a cellular mechanism (Mukhopdhyay and Ghose, 1976).

1.1.4 Mechanisms of response to DO levels.

Harrison (1972 and 1976) has reviewed the mechanisms by which cells adjust to different DO levels a summary of which is presented here. Four levels of response over time can be distinguished in growing microbes.

Within seconds a primary response with no feedback control occurs where molecules in the organism undergo chemical or physico-chemical interactions with the change in the environment, following Michaelis-Menten kinetics. Very few changes occur this way.

After 15 minutes or less feedback control of metabolic pathways can occur, and are often dictated by changes in adenine nucleotide levels. The ATP/ADP ratio was established as an expression of energy status which would indicate overall metabolic direction, however it is not an accurate expression of available energy phosphate bonds since adenylate kinase catalyses a freely reversible reaction between 2 ADP and ATP + AMP, which acts to buffer the levels of these 3 molecules. Therefore, Atkinson's hypothesis was formulated to describe the effects of changes in adenine nucleotide levels on energy charge (EC) (Chapman *et al.*, 1971); the resulting EC describes the cell's metabolic state. Energy charge is defined as (ATP + ½ ADP) / (ATP + ADP + AMP) (Atkinson and Walton, 1967). Decreases in EC favour catabolic processes generating more high-energy bonds as ATP, and rises in EC favour anabolic processes that utilise the high-energy bond. Oscillations are characteristic of feedback-controlled systems, and have been shown to correlate to changes in O_2 conditions in some bacterial fermentations. However Buchanan *et al.* (1987) showed adenylate energy charge had no effect on aflatoxin production by *Aspergillus parasiticus*, instead the cause was an apparent catabolite repression effect on Krebs cycle enzyme activity, through inactivation of the mitochondria.

On a time-scale of 1-10 hours responses occur through induction and repression of protein synthesis. Changes between anaerobic and aerobic conditions can cause considerable adaptations in the microorganisms; for example mitochondrial structure changes in yeasts (Visser *et al.*, 1990). A large number of enzymes in cells are regulated by O_2 availability, and the induction/repression of the synthesis of these may depend on concentrations of O_2 lower than the critical concentration required for respiration. Whether or not O_2 acts directly as an effector molecule has not been established, it may possibly alter the redox state of specific redox couples or of an effector molecule within the cell.

The final level of metabolic response (which is most relevant to bacteria) is at the selection of mutants on a time-scale of many generations. Continuous selection occurs in chemostat

cultures, where changes in DO cause strains of organisms more fit for the conditions to predominate.

1.1.5 Influence of redox potential on microbial cultures.

Redox studies in fermentations presuppose a very low DO concentration in the medium and O_2 acts as an oxidant. The redox potential (E_h) was proposed as a potential control in fermentation processes in which optimal product formation occurs at some point below full aerobiosis. E_h is defined by the Nernst equation, which is used to calculate electrode potentials or cell potentials for concentrations and partial pressures other than standard state values. Redox probes have been used to define what the E_h of a fermentation might be, but the result cannot be defined in terms of known electrochemical reactions. Observed correlations between metabolism and E_h changes could possibly be explained by changes in DO tension or substrate levels in the media. Direct fluorimetric measurements of NADH have shown that high frequency oscillations can occur, and levels respond to changes in the O_2 supply, especially when at low O_2 tensions. This type of experiment is considered a much better tool with which to observe the intracellular redox state, and key redox couples (Mukhopdhyay and Ghose, 1976; and Harrison, 1972). It has been considered that the overall NAD/NADH ratio or redox potential of a cell could be key to regulatory processes, but has been dismissed as being of little value by Harrison (1972) in favour of responses of electron acceptors and metabolic products to changes in O_2.

1.1.6 Oxygen and yeast metabolism.

Yeasts (unicellular fungi of the class Hemiascomycetae) have been the subject of many studies regarding the effects of O_2 on their metabolism. Four effects have been determined which describe O_2-related physiological phenomena depending on the species and on the sugar substrate.

The Pasteur effect describes the inhibition of fermentation in the presence of O_2, resulting in higher sugar uptake in 'fermenting cells' than respiring cells (Fiechter *et al.*, 1981). The effect occurs in resting cells of *Saccharomyces* species, and the increase in respiration rate caused by

anaerobiosis to increase ATP production is not due to increased respiratory capacity, but the loss of fermentative activity.

The Crabtree effect differs from the Pasteur effect because it is the substrate (eg. glucose or fructose) which causes repression of respiratory activity under aerobic conditions in growing cells. It is divided into short-term and long-term effects. The long-term effect refers to oxidoreductive (or respiro-fermentative) growth during steady-state conditions in aerobic glucose-limited chemostat cultures where the specific growth rates are above D_{crit} (the switchpoint between purely oxidative and oxidoreductive yeast growth), and where ethanol production remains constant. The short-term Crabtree effect is a direct reaction to a 'glucose pulse', causing the immediate production of ethanol and acetate (Van der Aar *et al.*, 1990). Pyruvate decarboxylase has been shown to be activated after the 'glucose pulse' in response to increased pyruvate levels, and competes with the mitochondria for it (Van Urk *et al.*, 1989). The Crabtree effect has been considered to be a stabilisation rather than a repression of respiratory activity since the k_La remains at constant high levels at growth rates above D_{crit}.

The third effect is the Custers effect, where fermentation is inhibited by the absence of O_2 in some yeasts eg. the *Brettanomyces* genus (Weusthuis *et al.*, 1994). It is considered a negative Pasteur effect and has been attributed to an initial shortage of NAD brought about by the activity of redox systems in the cell after the addition of glucose. The effect can be reversed by the addition of various carbonyl compounds and re-oxidation of NADH to NAD^+ (enzymatically and with O_2, and through the respiratory chain) so that it can participate in fermentations. Gaunt *et al.* (1988) relieved the effect with organic hydrogen receptors which restored the redox balance, although this cannot be explained in terms of the nicotinamide nucleotide pool.

The Kluyver effect is the fourth and least understood effect related to O_2 in yeasts, and occurs in over 40% of yeasts that ferment D-glucose anaerobically. It was originally defined by Sims and Barnett (1978) as occurring when certain yeasts utilise particular disaccharides aerobically, but not anaerobically, although under anaerobiosis the component monosaccharides were utilised. Weusthuis *et al.*, (1994) redefined it as the inability to ferment certain disaccharides to ethanol or CO_2 even though the respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexoses can occur. Accordingly,

this definition suggests that the Kluyver effect is not dependent on the O_2 concentration, but reflects an inability to metabolise the disaccharide fermentatively. Sims and Barnett (1978) concluded that the Kluyver effect resulted from the requirement for O_2 by sugar transport. In later work (1991), these authors showed that pyruvate decarboxylase activities were reduced when grown on the disaccharide. Pyruvate decarboxylase is involved in metabolic regulation in yeast and is necessary for glycolysis to proceed under anaerobic, but not aerobic conditions (Sims and Barnett, 1991). Other authors (Oura 1974; van Urk *et al.*, 1989) have implied the role of this enzyme, which plays an essential role in glycolytic regulation under aerobic conditions. Along with its observed role in the short-term Crabtree effect (Van Urk *et al.*, 1989), this enzyme can be considered an important switchpoint in mediating the various O_2 effects observed in yeasts. Weusthuis *et al.* (1994) indicated that there were 3 possible reasons the Kluyver effect could be caused by differences in monosaccharide and disaccharide metabolism: sugar transport, disaccharide hydrolysis, and sugar-specific regulatory mechanisms which occur before the level of pyruvate (reiterating Sims and Barnett's (1978) conclusions). The explanation proposed by Weusthuis *et al.* was that there is possibly a mechanism which tunes disaccharide uptake and hydrolysis in response to O_2 concentration or redox potential.

In studies unrelated to the 4 effects mentioned above, a series of 4 papers by Bruver (Bruver *et al.*, 1975a, 1975b) and Ball (Ball *et al.*, 1975a; 1975b) showed different possible means of regulation between cultures utilising glucose or galactose as the respective carbon sources. Using an O_2 challenge to anaerobic cultures, different responses to step-down transfers and anaerobic to aerobic transitions were noted. It was proposed that adenine nucleotides controlled the metabolism of glucose cultures, and that the NAD^+/NADH levels controlled galactose cultures, based on the enzymes induced by these hexoses in the anaerobic context. Phosphofructokinase was the rate-limiting step for glucose cultures since NAD^+/NADH cycling would not affect activity. The NAD^+/NADH cycling control in galactose cultures was thought to work by failing to induce alcohol dehydrogenase (ALDH) responsible for recycling, and/or by failure to induce pyruvate decarboxylase, so there would be no substrate for ALDH. The inability of galactose to induce glycolysis at the point of pyruvate to ethanol preconditioned the anaerobic cells, determining the subsequent response to O_2 challenge. In the glucose culture O_2 challenge resulted in a small amount of induced TCA activity (therefore the energy generating system was unaffected), by comparison the galactose culture

had pre-formed TCA activity facilitated by O_2, which out-competed the poorly-induced ethanol branch for pyruvate, resulting in decreased ethanol production. A 'control by induction' model for glucose regulation was proposed rather than other catabolite repression models.

1.1.7 Regulation of gene expression in yeasts by O_2.

Many yeast genes are differentially expressed in response to O_2. During oxidative growth more than 200 genes involved in respiratory functions, and the control of oxidative damage are induced. At low O_2 tensions hypoxic genes are expressed to allow the cell to utilise limiting O_2 more efficiently, these include cytochrome subunits and oxidases, and desaturases in heme, sterol and fatty acid biosynthesis. Zitomer and Lowry (1992) published an extensive review of the mechanisms involved, and this is summarised in the following paragraphs.

Heme is one molecule which plays a significant role in O_2 regulation. It is a prosthetic group in cytochromes and some O_2-binding proteins such as catalase, as well as acting as an intermediate in the signalling mechanism for O_2 levels in yeast cells. Heme biosynthesis has an essential requirement for O_2 at 2 enzyme-mediated steps where it acts as an electron acceptor, one of which is the rate-limiting step for heme biosynthesis, and is therefore controlled by O_2 tension. The remaining pathway enzymes do not become functional until the presence of heme is detected.

There are 2 categories of heme-regulated genes: heme-activated and heme-repressed. Heme-activated genes fall into 2 main groups-those encoding respiratory functions (eg. cytochrome units) and those encoding oxidative damage repair functions (eg. catalase, Mn-superoxide dismutase). The heme-repressed genes are the hypoxic genes which function in the utilisation of O_2 in electron transport, or in membrane or heme biosynthesis gene products which have no purpose in cells growing in complete absence of O_2.

Two distinct heme activation protein complexes have been identified: *HAP1* and the *HAP2/3/4* complex and at least 4 genes are activated by both. The HAP1 protein binds to upstream activation sites (UASs) of a number of genes to activate transcription in the presence of heme. DNA binding is masked in the absence of heme. The HAP2/3/4 complex responds

to 2 stimuli for transcriptional activation to occur-heme and non-fermentable energy sources (eg. lactate added as the carbon source instead of glucose). Some genes experiencing this glucose catabolite repression effect are activated by HAP1 to overcome this. All 3 units are required for activation. HAP4 is the transcriptional activation domain, HAP2 is probably the DNA binding region. The HAP2/3 heterodimer acts as a general transcription factor in the regulation in a variety of cellular functions and is believed to associate with different genes by using alternate HAP4-like subunits as the regulatory mechanism requires.

The Rox1 repressor is responsible for heme repression of gene expression. It acts at 2 operator regions with the consensus sequence YYYATTGTTCT which lie between the TATA box and the UAS region, in a manner parallel to the activation of genes coding other translational components. Heme transcriptionally activates *Rox1* gene expression partially due to HAP1 and HAP2/3/4, however it is not affected by catabolite repression like other genes regulated by this complex. Heme repression of gene expression by Rox1 occurs only at transcription of the *Rox1* gene. Represson by Rox1 requires 2 other proteins Tup1 and Ssn6 in a complex which serves as a general repressor, and it is thought the function of Rox1 is to anchor the Tup1-Ssn6 complex to specific genes targeted for repression since it contains an HMG box found in other sequence specific binding proteins in its N terminus, there is also a C terminus domain which binds the Tup1-Ssn6 complex (Deckert *et al.*, 1995).

Heme-independent O_2 regulation has also been found in yeasts in the form of 2 systems-mitochondrial gene regulation and anaerobic genes. Mitochondrial DNA encodes functions for its maintenance, and the sub-units of the oxidative phosphorylation apparatus-these genes are regulated by O_2. One example is *PET494*, a nucleus-encoded factor which works with 2 other factors (PET122 and PET54) to recognise the 5' leader of CoxIII mRNA (cytochrome *c* oxidase subunit), and it is regulated at the level translation in a O_2-dependent, heme-independent manner.

Anaerobic genes (*ANB2-ANB15*), of which 4 are identified as 'early' and 11 as 'late' (ie. they require at least 3 hours anaerobiosis), are expressed exclusively in the absence of O_2. The early genes *ANB13*, *ANB14* and *ANB15* were heme and Rox1 independent in action, as was the induction of 4 of the late genes. These anaerobic genes could be more sensitive to low O_2 levels than heme-repressed genes.

1.2 OXYGEN AND CARBON METABOLISM.

The effects of O_2 on carbon metabolism in hyphal fungi were investigated by Woodhead and Walker (1975) who studied the effects of aeration on glucose catabolism in *Penicillium expansum*; this thesis is based upon much of that work. Liquid cultures of *P. expansum*, aerated by shaking and grown in a malt extract medium, showed considerable differences in enzyme activities and isozyme patterns compared to those observed in static cultures. In particular, the activities of 2 pentose phosphate (PP) shunt enzymes, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), were elevated in shake cultures, reflected in the multiple bands present on isozyme gels. Both the enzyme activities and the number of isozymes decreased as the fungal metabolism entered idiophase later in the fermentation. By contrast, static cultures exhibited minimal PP enzyme activities, with an extra isozyme not appearing until after 96h for both enzymes, leading to the assumption that perhaps the PP shunt is not utilised until idiophase in static cultures. The polyketide secondary metabolites patulin and citrinin were produced in large amounts in the static cultures, which was consistent with other studies that have indicated that polyketide production occurs when there is a lack of NADPH (used in reductive processes) produced by the PP shunt. In the presence of NADPH acetyl CoA would normally be diverted into fatty acid biosynthesis but would be utilised instead by the polyketide synthases in these conditions. A fuller explanation of secondary metabolism and patulin biosynthesis follows later in Chapters 2.1 and 5.1.

Glycolysis was important and occurred at the same rate in both shake and static cultures. The TCA cycle operated in both, but succinate dehydrogenase (SDH) activity was higher in shake cultures. The SDH isozymes behaved in a similar way to the PP shunt isozymes; in the shake cultures these were greatest in number during tropophase then decreased in idiophase, whereas in the static cultures these increased in number during idiophase.

Another important observation in these studies was that extracellular pectolytic enzyme activity was greater in shake cultures. The transamination role played by the TCA cycle, and its increased activities in shake cultures, together with the increased pectolytic activity lead to the conclusion that in shake cultures the production of pectolytic enzymes represented a diversion away from secondary metabolite production. The increased pectolytic enzyme

activity could have been a response to more readily available and utilisable growth substrates for the fungus through the increased aeration levels.

Dijkema *et al.* (1985) took a different approach to studying carbon metabolism in *A. nidulans*. ^{13}C NMR was used to monitor the levels of various polyols, TCA intermediates, amino acids, and phospholipids present during growth on glycolytic (sucrose or sucrose/acetate) or gluconeogenic (acetate only) carbon sources under different conditions of aeration. Polyols are low molecular weight sugar alcohols forming endogenous reserves that are continuously metabolised even in the presence of exogenous substrates, with pool sizes and compositions varying according to stages in the fungal life cycle. Polyol resonances were found to dominate the sugar resonance region of the ^{13}C NMR spectrum. The nature of the carbon source affected the pool sizes of the various polyols, and O_2 had an important effect on glycolytically grown cultures. O_2 stimulated flux through the PP shunt and glycolysis, the ratio of erythritol (via the PP shunt) to mannitol (glucose-derived) increased, and there was an overflow of reduced metabolites at the PP shunt (ie. of erythritol and arabitol) and at the C_3 level of glycolysis (glycerol). Gluconeogenic substrates did not stimulate the PP shunt. The TCA and amino acid resonance region of the spectra showed pronounced glutamate and glutamine concentrations when vigorous aeration was employed, reflecting high TCA cycle activity. The results of both studies lead to the common conclusion that O_2 plays a very important role in stimulating PP shunt activities and the TCA cycle. Both these pathways are important in regulating other metabolic functions.

1.3 AERATION AND FERMENTATIONS

O_2 has been used as a factor controlling the production outcome of fermentations since it can influence the metabolism of an organism of interest. The following examples show how aeration can affect a target biochemical such as commercially or potentially important enzymes and antibiotics, as well as demonstrating the importance of such metabolic studies to improve fermentation efficiencies.

1.3.1 Products of primary (or intermediary) metabolism.

Citric acid (a TCA cycle metabolite) production from *A. niger* is one example of a commercially important fermentation, and overproduction of this has been achieved by using the specific O_2 uptake rate to control the intracellular ammonium level (Kim *et al.*, 1991). Metabolic studies on this organism showed that intracellular NH_4^+ ions (NH_4^+i) were potent regulators of the phosphofructokinase (PFK) enzyme in glycolysis – low levels of NH_4^+i resulted in increased citric acid production, especially once the culture was in idiophase. Increases in NH_4^+i lead to an increase in the specific O_2 uptake rate. The optimal value for NH_4^+i was 3-4.5 mM/g cell, concentrations higher than this level would limit citric acid production. It was proposed that sub-optimal concentrations of NH_4^+ ions would be necessary for cellular maintenance, and possibly counteract the feedback control of citrate at the PFK step, providing a mechanism for continued citric acid production.

A point to note when studying the role of a physical parameter such as aeration is that not only O_2 transfer processes are involved. Dissolved CO_2 concentration and its effects on citrate production by *A. niger* A60 was studied by McIntyre and McNeil (1997). CO_2 is believed to be inhibitory to the citric acid production process, and would be especially relevant to such a significant industrial process. The role of CO_2 in the inhibition of growth and metabolism or the specific CO_2 species involved is unclear. Below pH 4.0, the only major species present is $CO_{2(aq)}$. In this study *A. niger* A60 growth was inhibited with continuous gassing at all concentrations studied, and with increased CO_2 concentration the production of citric acid decreased.

The morphology of the organism also changed: the pellet diameter increased due to the formation of long hyphae around the pellet edge, which is unlike the short, stubby hyphae which are normally associated with citrate production. The mechanisms weren't defined by this study however there were some possibilities offered. Alteration of substrate utilisation, altered enzyme equilibria, specific effects on TCA and glyoxylate cycle enzymes (through a change in pH or similar properties) and altered morphology of *A.niger* were all considered possible causes.

Production of another TCA cycle-derived metabolite, itaconic acid (methylene succinic acid), was O_2 dependent. Itaconic acid is a specialised monomer used as a copolymer in commercial acrylic resins and polymers, and in *Aspergillus terreus* is formed by decarboxylation of *cis*-aconitate from the TCA cycle. Itaconic acid biosynthesis is dependent on media composition, and is strongly influenced by physico-chemical and biological factors. The fermentation pH must be rigidly controlled at 1.8-1.9 and continuous aeration is required. Gyamerah (1995) showed *A. terreus* to be sensitive to a lack of O_2 of more than 5 minutes and metabolic inhibitors of ATP synthesis in shaken submerged cultures. The O_2 dependence involved protein synthesis for production. ATP synthesis was required to maintain the fermentation, and possibly to maintain a normal physiological pH in the acidic fermentation despite ATPase having no effect on preventing acidification of the cytoplasm (the plasma membrane may have been impermeable to H^+ ions). The role of ATP might be an involvement in the transport of itaconic acid from the cell.

Lazic *et al.* (1993) studied the effects of pH and aeration on extracellular dextran production by *Leuconostoc mesenteroides* in order to optimise conditions used industrially for this process. Dextran is produced from sucrose by dextransucrase and occurs simultaneously with growth. The best yields of dextran and stability of the dextransucrase occurred when the O_2 transfer rate was equal to the maximum O_2 uptake rate of the organism. Under anaerobic conditions the fructose and glucose formed by dextransucrase are utilised by the organism as an energy source, and at higher than optimal aeration the glucose portion is utilised rapidly by the organism, reducing dextran yields.

Kojic acid is produced by *Aspergillus flavus* Link and has many industrial applications including medical use as an anti-inflammatory drug and painkiller, as a precursor for flavour enhancers in the food industry, and cosmetically it is used as a whitening agent. The biosynthesis of kojic acid is believed to be mediated in part by enzymes of primary metabolism such as glucose-6-phosphate dehydrogenase, hexokinase, and gluconate dehydrogenase, and an O_2 requirement is considered essential in the early stages of the fermentation since these enzymes are very important in glucose catabolism. Ariff *et al.* (1996) have studied the optimum aeration requirements for this fermentation and shown that the fermentation required two phases: growth and production. Optimal kojic acid production occurred when DO was controlled to 80% in growth phase, and at 30% in production phase

where the mycelial growth was reduced provided no yeast extract or external N source was added, which resulted in the inhibition of production.

Pullulan is an extracellular polysaccharide of *Aureobasidium pullulans*, formed from α-(1→6)-linked maltotriosyl residues with an occasional random maltotetriosyl substitution. This polymer is considered useful in a number of medical and industrial applications. Pullulan production by this fungus reduced with increasing agitation rates, and was shown to due to increases in pO_2 in the medium and not increases in shear forces. The length of time that the fungus is exposed to low pO_2 conditions to improve production is believed to be critical, however no mechanisms for regulation were described (Gibbs and Seviour, 1996).

1.3.2 Enzyme production.

The effect of O_2 on growth and glucose oxidase production in *A. niger* was studied by Zetelaki and Vas (1968). Increases in both these parameters were observed when increases in agitation rate were applied to a 10 L fermentor culture, with further improvements achieved by using pure O_2 instead of air. Viscosity of the culture increased with the increased concentration of mycelium that resulted, and decreased with the appearance of autolysis, which occurred more rapidly in these cultures also. Increases in sugar concentration in the more highly aerated cultures did not affect the growth and enzyme production rate, indicating that O_2 was the limiting and therefore controlling factor of the fermentation.

Another enzyme of interest produced by an *A. niger* strain is glucosyltransferase, an enzyme which produces iso-malto-oligosaccharides (IMOS) which are used as low calorie and non-carcinogenic sweeteners, and for the improvement of intestinal microflora by acting as a growth factor for Bifidobacteria. IMOS are produced by glucosyltransferase by the transfer of non-reducing D-glucose residues from many types of donor substrates to suitable receptors. The results showed that maximum production of this enzyme occurred at an initial maltose concentration of 40 g/L, and at an agitation rate of 750 rpm and an aeration rate of 1 L/min in a 5 L fermentor. This fermentation was dependent on a high DO concentration (Chen *et al.*, 1996).

Plant cell wall-degrading enzymes are important in processes such as kraft pulp bleaching and the degradation of resistant polymers such as lignin and related chlorinated compounds. Several studies have been undertaken to optimise the conditions for the production of these enzymes, and O_2 levels have been shown to play an important role in these processes. The cellulolytic enzymes of *Trichoderma reesei* QM9414 (a mutant for cellulolytic enzyme production), xylanase and endoglucanase, were studied along with growth and respiration under various agitation rates on 2 different substrates-lactose (for growth studies) and Avicel (a microcrystalline cellulose, for enzyme studies) (Lejeune and Baron, 1995). Pellet formation occurred in this fungus at all agitation levels for both substrates, but high agitation rates resulted in decreased enzyme production. Enzyme production in this fungus required growth to be limited and the hydrolysis of Avicel to have occurred.

Brandão Palma *et al.* (1996) studied the effects of agitation and aeration rate on xylanase production in *Penicillium janthinellum.* This enzyme degrades xylans in the hemicellulose fraction of plant cell wall material. Xylanase synthesis was associated with growth and shown to be a function of k_La so that greater production occurred at low aeration and low agitation rates, and was more successful in shake flasks than in a stirred tank fermentor. This response to aeration and agitation was believed to occur because the process was sensitive to mechanical shear. A degree of agitation was deemed necessary to maintain medium homogeneity and avoid formation of large metabolically inactive pellets, however large shearing forces would disrupt mycelial tissue and therefore xylanase production. The inhibitory effect of O_2 on xylanase production was attributed to inhibition or inactivation of enzymes.

The formation and degradation of the ligninolytic system of *Phaenerochaete chrysosporium* was markedly affected by O_2. The production of extracellular proteases and polysaccharides was high with continuous oxygenation, and correlated to low levels and fast decay of lignin and manganese peroxidases (LiP and MnP) (Dosoretez *et al.*, 1990). The nature of the O_2 supply affected the patterns of appearance of these enzymes; a continuous or periodic supply of air maintained MnP supply but lignin peroxidase was undetectable whereas a periodic supply of O_2 resulted in slower decay of LiP and a continuous supply of O_2 resulted in a rapid decrease in LiP activity.

1.3.3 Antibiotic production.

The effects of O_2 on antibiotics produced by the filamentous prokaryotes, *Streptomyces* have been well-studied, however studies on eukaryotic fungi are few. One such study involved the use of miniature electrodes fitted into the sides of shake flask cultures of *Saccharopolyspora erythraea* and *Amycolatopsis orientalis* (Clark *et al.*, 1995). Erythromycin produced by *S. erythraea* paralleled biomass production, and was produced under both O_2-limitation and glucose-limitation, whereas in *Amy. orientalis* vancomycin production required O_2 and occurred after growth slowed. The *S. erythraea* cultures also produced an unidentified red pigment in O_2-limited cultures which was proposed to be an 'overflow' metabolite of some type since the carbon source was still being assimilated.

Virgilo *et al.* (1964) showed that oxygenation was necessary for rifamycin production by *S. mediterranei* by measuring DO, O_2 diffusion rate, dry weight, viscosity, pH and sugar utilisation for different impeller diameters, speeds and airflows. 50-80 hours was identified as a critical time during the fermentation, during which high agitation speed resulted in high antibiotic production since the O_2 demand of the organism was being met.

Other studies have shown O_2 to have a positive effect on antibiotic production. Rollins *et al.* (1988) studied cephamycin C production in *S. clavuligerus* and showed that the fermentation required near saturating levels of DO to maintain production without affecting growth rate. Reduced oxygenation possibly limited the availability of antibiotic precursors since precursors would be directed through the lysine pathway into proteins rather than to the ACV synthase, the first enzyme of the penicillin/cephamycin pathway. Similarly, amino acid pools may have been starved in such conditions, affecting amino acids involved in cephamycin C production such as methionine, which stimulates ACV synthase, cyclase and expandase. This study was performed in 10 L fermentors, and difficulties in controlling DO concentrations were expected in scaling-up the fermentation.

Positive effects of O_2 on antibiotic production have also been noted in *Streptomyces galilaeus* strains producing anthracyclines (Královcová and Vanek, 1979), and in colabomycin-producing *S. griseus* (Dick *et al.*, 1994). In *S. galilaeus* the means of agitation had a considerable effect on the morphology and therefore the biosynthetic activity. Pellets were

formed when a reciprocating shaker was used, whereas a rotary shaker produced filamentous mycelium. Pellet formation resulted in decreased O_2 transfer and therefore galirubin production. Colabomycin production by *S. griseus* was believed to be a result of the normal pathway of substrate utilisation involving glyceraldehyde-3-phosphate dehydrogenase, being bypassed via fructose-1-phosphate and glycerate, which may have caused an increase of precursors feeding into the colabomycin polyketide synthase. Several studies have indicated the greater desirability of controlling DO and aeration parameters than mutant selection processes to industry.

O_2 can affect the patterns of antibiotics produced in a given organism. In another study on *S. clavuligerus* fermentations (Rollins *et al.*, 1990), O_2 was shown to derepress deacetoxy-cephalosporin C synthase (DAOCS), the enzyme responsible for converting penicillin N to cephamycin C. Under O_2-saturating conditions DAOCS activity increased 2.3-fold compared to the penicillin ring-cyclising enzyme, isopenicillin N synthase, which only increased 1.3-fold. The quantitative ratio of cephamycin C production to total antibiotic production gave a measure of the comparative efficiencies of the 2 enzyme systems. O_2 also affected the time of appearance of cephamycin C. The mechanisms for derepression are unknown.

The pattern of antibiotic production by *S. fradiae* was also affected by O_2. The organism used was a stable mutant producer strain (NRRL 2702) which produced tylosin, a macrolide antibiotic. At high DO levels and high aeration rates an additional macrolide antibiotic, macrocin, was synthesised. Macrocin is produced on the biosynthetic pathway leading to tylosin, and its production was concluded to be due to the enhancement of enzyme activity by the conditions rather than repression or inhibition of the enzyme responsible for converting it to tylosin. It was also inferred that cofactors and precursors would not have been limiting since this appeared to be the rate-limiting step of this mutant (Chen and Wilde, 1991).

One bacterial fermentation showed that O_2 can interfere with antibiotic production. *Bacillus brevis* required O_2 for growth to occur, but gramicidin S production was inactivated by its presence. The gramicidin S synthetase complex (which catalyses non-ribosomal protein production) required stabilisation by N_2, since the rapid disappearance of this enzyme at the start of stationary phase was attributed to O_2 (Friebel and Demain, 1977).

CHAPTER 2

SECONDARY METABOLISM

2.1 INTRODUCTION

Secondary metabolism refers to a series of anabolic processes that occur within an organism and are not considered to be essential for the organism's replication and growth. The products of secondary metabolism (idiolytes), can occur uniquely in a single strain or species, in two closely-related members of a single genus, or may be found sporadically in a limited number of evolutionarily-unrelated species of different genera, families, orders, classes, phyla, or kingdoms (Campbell, 1983). Secondary metabolites accumulate mainly in particular organs or tissues of plants, and microorganisms (Haslam, 1986), and include a wide range of chemical structures. Some of these structures include special chemical groups such as covalently-bound chlorine and bromine; nitro, nitroso, isonitrilo, diazo and hydroxamic groups; phosphono, phosphino, phosphonamido; unusual ring systems such as β-lactams, tropolones, dioxopiperazines, macrolides, xanthones, isochromaquinones (Zahner, 1979). They include amino sugars, quinones, coumarins, epoxides, ergot alkaloids, glutarimides, glycosides, indole derivatives, lactones, macrolides, naphthalenes, nucleosides, peptides, phenazines, polyacetylenes, polyenes, pyrroles, quinolines, terpenoids, and tetracyclines (Martin and Demain, 1980).

The biosynthesis of secondary metabolites is generally directed by organised sets of genes associated with special regulatory mechanisms that control both the timing and level of gene expression (Vining, 1992). These control mechanisms are well-integrated with the physiology of the producing organisms. Many secondary metabolites have been found to be growth-inhibitory, toxic to other organisms, and the cause of allelopathy in plants (ie. chemicals which inhibit the growth or reproduction of other plant species with which the producer is competing) (Haslam, 1986). Possible roles for these and their significance are discussed later

in this section. Secondary metabolites have been referred to as idiolytes, shunt metabolites, special metabolites or 'chemists' compounds' (Campbell, 1983).

By contrast, primary or intermediary metabolism (primary metabolites are also known as 'biochemists' compounds') is concerned with the pathways for energy production which make up the central processes for cellular survival. Primary metabolism involves an interconnected series of enzyme-mediated catabolic, amphibolic and anabolic pathways which provide biosynthetic intermediates and energy and convert biosynthetic precursors into essential macromolecules in processes identical for all living things (Martin and Demain, 1980). Therefore, this involves the compounds found in most organisms which are considered normal cellular constituents. A series of kinetically-controlled enzyme reactions link primary metabolism, and function with very low standing concentrations of primary metabolites which are therefore unlikely to accumulate. Primary processes tend to be governed by substrate levels whereas secondary processes are limited by their enzymes (Haslam, 1986).

The boundary between primary and secondary metabolism is ill-defined. Compounds exist which appear to be essential metabolites, however there is no conclusive evidence to support any of their proposed cellular functions. This may reflect a lack of knowledge of the biological function of secondary metabolism. Many secondary metabolites have acquired a physiological role in inter- and intra-specific interactions of organisms; examples of these are pheromones (chemical substance emitted by one organism as a signal to another) and phytoalexins. There are also primary metabolites which can be classed as secondary metabolites because of the nature of their production. An example of this is the overproduction of citrate by *Aspergillus* species which appears to have no cellular function and therefore is classed as a secondary metabolic event (Garraway and Evans, 1984). There are several theories which attempt to explain the existence of secondary metabolism and it is generally assumed that secondary metabolism must have been of some selective advantage to the organism at some stage during its evolution. The following is summarised from Haslam (1986).

The first category for theories for the existence of secondary metabolism is that at some stage in the life of the organism the secondary metabolites had, or still have, some functional or metabolic role. A second group of explanations view secondary metabolites as waste or

detoxification products. Thirdly, secondary metabolism may be considered a measure of the fitness of an organism to survive since there are compounds which attract or repel other organisms as part of that organism's strategy for survival. The final category is that secondary metabolism may allow organisms to adjust to changing circumstances. The synthesis of enzymes in secondary metabolism permits the network of primary enzymes to continue to function until circumstances allow renewed metabolic activity and growth. This means that the processes of secondary metabolism rather than the products are important, and it could be possible that secondary metabolites have subsequently acquired a functional role.

Several ideas have been proposed based on the fourth hypothesis. Woodruff (1966) proposed that secondary metabolites are 'shunt' metabolites, which serve to reduce abnormal concentrations of normal cellular compounds (which are sometimes toxic at such levels). Shunt metabolites form when other pathways for that intermediate are wholly or partially closed when growth ceases (Bu'Lock, 1961), and therefore high pool levels of intermediary metabolites accumulate. Shunt metabolism results in the modification of normal pool metabolites, which tend to undergo condensations and polymerisations. The process of secondary metabolism is more essential than the products because secondary metabolism allows the uptake of nutrients to continue, depriving competing organisms of nutrients not already exhausted. Bu'Lock and Powell (1964) suggested that secondary metabolism serves to maintain basic metabolism in circumstances when its normal substrates through depletion of nutrients can no longer be exploited for normal cellular growth and replication. The advantage to the organism occurs with the rapid renewal of growth when favourable conditions are resumed. Metabolic intermediates normally present at low concentrations accumulate, leading to induced formation of new enzymes to relieve the pressure of key metabolic intermediates. Another idea is that secondary metabolites are waste products of general metabolism in phylogenetically less-advanced organisms (Haslam, 1986).

The sophistication of the biosynthesis, regulation and genetic organisation leads to the assumption that secondary metabolism is beneficial to the organism, and that it would have been eliminated if it had not been maintained by selection. This rationale overcomes the argument that secondary metabolites are metabolic refuse and that detoxification, storage, metabolic idling or shunting metabolic overloads instead provide the overall rationale for the process (Vining, 1992). Some plausible hypotheses relate to signalling, differentiation,

sequestering nutrients from the environment, mediating ecological interactions, and facilitating biochemical evolution. The large majority of secondary metabolites are biologically active with two principal themes for their function: to serve a purpose within the producing species such as mediation of growth, reproduction or differentiation, and to act on targets external to the producer. Examples of this are the production of mycotoxins, antibiotics, insecticides, herbicides (ie. competitive interactions), some of which appear unnecessary to the producer organism (Vining, 1992). Woodruff (1966) suggested that antibiotics do not confer a survival advantage if antibiotic production is considered the normal situation in microbial growth due to limitations in the environment, and that antibiotics are not produced to the same extent in nature in the context of microorganisms living with and competing against each other. The significance of secondary metabolites reflects the role of ancestral low molecular weight substances in facilitating primordial biochemical reactions. It is possible that secondary metabolites originally functioned as shunt metabolites or as a metabolic step to allow the continued functioning of primary metabolism, but over time developed into part of the defense armoury of the organism.

As well as there being several theories for the reasons for the existence of secondary metabolism, there are also 3 categories of theories for the evolution of such biosynthetic pathways. Subtractive theories make up the first group. These are based on the idea that an ancestrally inefficient, but potentially versatile organism evolved more discrete pathways by the development of increased specificity. Opposing these are the accumulative theories in which extant biochemical pathways are presumed to have arisen from simpler ancestral pathways. Retrograde evolution theories make up the third group, where a backwardly-evolving pathway recruited new enzymes in reverse order (Haslam 1986). The primitive enzymes of ancestral cells probably possessed very broad specificities in order to exploit a range of resources, prior to nitrogen being introduced to metabolism. The main energy production pathways of the cell, glycolysis and the TCA cycle, do not contain N. Retroevolution, involving the antecedents of secondary metabolism, could be responsible for shaping the metabolic systems and pathways responsible for their biosynthesis, however all secondary metabolic pathways originate at a precursor in the primary metabolic network (Vining, 1992).

Vining also discusses theories which are based on the idea that individual secondary metabolic pathways developed as a result of chance doubling and subsequent mutation of a gene that directed the synthesis of a primary metabolic enzyme, so that the altered copy acted on the substrate to give an abnormal product. The product may become useful to the organism after spontaneous chemical change, or by resident enzymes of relaxed specificity. Random modifications might have occurred through several steps before chance formation of a beneficial product resulted in a strain that possessed a selective advantage. Gradual improvement of this pathway would have consolidated the benefits conferred by it and ensured maintenance of genetic information. Therefore secondary metabolism would have developed stepwise in a forward sequence from a pre-existing primary network that provided the essential metabolic needs of the organism.

Zahner *et al.* (1983) proposed a 'biochemical playground' concept where secondary metabolism operates alongside the five primary cellular events of intermediary metabolism, regulation, transport, differentiation and morphogenesis, and provides a place for continual biochemical evolution. The existence of this playground depends upon the supply of surplus precursors and energy from intermediary metabolism, and secondary metabolism allows the evolution of biochemical pathways to proceed in all directions provided the metabolites are not toxic to the producer at the time of their appearance. Continuous evolution of pathways in secondary metabolism occasionally leads to compounds which offer an advantage to the producer organism, and once developed become a part of primary metabolism. The functional equivalence of structurally different compounds is an important feature of the postulated 'playground'. Once a useful biochemical pathway has been invented in secondary metabolism, it can be utilised by other organisms. The symbiont theory of eukaryotic evolution is seen as an example of this, where pre-existing information was utilised because it saved time over *de novo* synthesis of genetic information. The transfer of genetic material occurs easily through symbiont-host and parasite-host type relationships, or through close contact of organisms in common habitats such as soil. Therefore communication of useful information by genetic transfer amongst different organisms becomes possible.

Comparisons have been made between secondary and primary pathway genes. One example is the similarity of polyketide biosynthesis to that of fatty acids, with the main differences lying in the partial or complete absence of reactions to reduce the β-keto group after chain

extension, and the larger variety of acyl precursors incorporated (Vining, 1992). Amino acid sequence similarities in the proteins which catalyse these reactions infer a common evolutionary origin; however the lack of similarity between polyketide synthase and fatty acid synthase from the same organism does not concur with the gene duplication and subsequent evolution theory (Wang *et al.*, 1991). Another example is that of the β-lactams; ACV (α-aminoadipyl-cysteinyl-valine) synthase catalyses the condensation of the three amino acids, α-amino adipic acid, cysteine and valine, in a manner which resembles the polypeptide synthases from *Bacilli*. Nucleotide sequencing confirmed an evolutionary relationship, however this holds no similarity to ribosomal polypeptide synthesis in primary metabolism and is therefore not a modification of existing primary metabolic pathway genes. Other enzymes involved in β-lactam biosynthesis have similar traceable origins and similarities amongst themselves or related processes in the pathway. It is also inferred that in this case entire gene clusters have been transferred because of the similarities of sequences, the lack of introns, and the persistence of gene clusters even though arrangements within these have subsequently changed around (Vining, 1992).

Industrially, secondary metabolism is a useful concept when predicting process characteristics since certain physiological events coincide with the initiation of product formation (Bushell, 1989). Three types of processes have been described (although the descriptions are considered simplistic and outdated):

Type I: formation of growth-associated products which are involved in the biosynthesis of daughter cells or energy metabolism associated with undifferentiated growth.

Type II: products formed from substrates involved in primary metabolism but via alternative pathways (growth-associated).

Type III: product formation occurs at an entirely different time to primary metabolism and are often derived from amphibolic pathways (go in reverse as well as forwards) rather than from catabolism.

Since secondary metabolism occurs in response to the concentrations and assimilation kinetics of many of the principal culture nutrients, the initiation of secondary metabolism may overlap with growth phase if a specific regulatory nutrient is depleted. If the nature of this regulation is known then secondary metabolism can be predicted and also manipulated, however this varies amongst different organisms and pathways.

Borrow *et al.* (1961, 1964) defined five distinct phases of growth in *Gibberella fujikuroi* (producing gibberellic acid) by varying concentrations of the key nutrients glucose, nitrogen, phosphorus and magnesium so that all four were exhausted at a selected dry weight in all possible sequences thereafter, and in other studies of other factors in the fermentation such as pH (initial and at nutrient exhaustion), agitation rate and airflow, and inoculum size. The productivity decreased with increasing glucose concentration and the maximal amount produced was proportional to the initial N provided.

Borrow's first phase is referred to as the 'Balanced phase of growth', a period when rapid growth and nutrient uptake extended from the start of growth until the first nutrient was exhausted (glucose and N had the greatest effects) so that an apparently preferred constant mycelial form was being produced in nutritionally unlimited conditions occurring at the initial specific growth rate until some change affects metabolism. Growth in the Balanced phase was defined to fit an exponential growth model. At least 5 metabolic states can follow the first period of exponential growth in N-limited fermentations: storage, exponential, decelerating, step and linear. Linear growth may in turn be followed by a form of decelerating growth, while the storage phase occurs after N exhaustion irrespective of the form of growth preceding this event. Linear growth is evidence of a response to O_2 restriction: increased mycelial growth results in decreased turbulence and rate of O_2 supply while demand for O_2 increases, therefore the onset of linear growth is the point at which demand exceeds supply. The rate at which exponential growth changed to linear growth was greater the higher the rate of agitation.

The second phase, Transition phase followed the balanced phase in the first study and was characterised by increased mycelial fat and carbohydrate content after exhaustion of the limiting nutrient. Proliferation of the fungus still occurred but the mycelial composition was changing from that observed in growth phase. This phase continued until reserves ceased to be used at which time the Storage phase began. During Storage phase, all nutrients except glucose ceased to be taken up from the medium, carbon and other reserves were accumulated in cells in which the components of the living matter were being reorganised after proliferation had ceased. Dry weight reached its maximum value during this phase. The Maintenance phase occurred when external or internal carbon sources were metabolised by

virtually unchanging living matter and occurred until no utilisable carbon remained. During the storage and maintenance phases the amount of living matter remained virtually constant, while the whole dry weight continued to increase during storage phase, so turbulence may continue to decrease. At this point the Terminal phase of mycelial breakdown started. This autolytic phase was characterised by increased vacuolation and loss of cell contents, and the return of NH_3, P, Mg, and K to the medium as well as an increase in medium pH to greater than 8.

Borrow's balanced growth phase is analogous to tropophase and the storage and maintenance phases are considered part of idiophase.

Regulation of secondary biosynthesis was studied in *G. fujikuroi* grown in batch and chemostat culture and the major features of batch culture development were related to the degree of limiting substrate depletion and the corresponding growth rate (Bu'Lock *et al.*, 1974). The major features of secondary metabolism in this organism were bikaverin (a polyketide, red, antiprotozoal quinone), gibberellin (terpenoid), and mycelial carbohydrate accumulation (which occurred with declining growth rate and is therefore believed to be a non-vegetative growth process). Six phases were described in the batch cultures regarding the growth and products of this organism. The bikaverin was produced earlier than the gibberellin, but both were in response to declining N concentrations. Thus production was controlled by a common mechanism of growth-linked suppression to which the two processes had differing sensitivities. Alterations to the dilution rates in chemostat cultures also resulted in differential synthesis of the 2 compounds at different rates; gibberellins at a lower rate than the bikaverins. Replacement cultures were also performed with mycelium being replaced onto N-free medium, resulting in bikaverin production, and when glycine was reintroduced production was stopped. Since this occurred only when RNA and protein synthesis were not inhibited, it appears the turnover of protein components was involved in the rate-limiting mechanism. Gibberellin synthesis was under the same type of control except exerted at a lower level of limiting nutrient and a corresponding lower growth rate. The exhaustion of the N source was believed to be the means by which this growth-linked suppression mechanism operated.

However studies by Grootwassink and Gaucher (1980) showed overlapping of the growth and production phases have been observed, due to a heterogeneous cell population. Their studies on the production of patulin by *P. urticae* showed that patulin required a carbon source, but the depletion of N triggered the production of this polyketide. They also showed that secondary pathway enzymes were synthesised *de novo*, sequentially prior to patulin production.

Secondary metabolism can be described as biochemical differentiation that may accompany morphological differentiation in a variety of fungi. It is a response to conditions not permitting further cell multiplication. The morphological response to form specialised structures (e.g. conidia, sclerotia) to ensure prolonged survival and resumption of growth at a later time is a more long term process, however there is evidence to indicate that the two processes are interrelated.

Differentiation can be described as the stable development of an altered structure or function, in fungi this occurs at the end of active growth phase resulting in a morphologically diverse array of structures. It is irreversible and excludes transient changes referred to as adaptations. Two mechanisms of gene expression are involved: in the short term the time at which initiation of differentiation occurs, and the commitment to pathways is dictated by nutrient conditions. In the long term, changes in enzyme composition of the cell are responsible for regulating differentiation mechanisms (i.e. under genetic control) (Martin and Demain, 1978).

The relationship between secondary metabolism and differentiation in fungi has been alluded to by a number of authors. Woodhead and Walker (1975) indicated there was potentially a relationship between 6-methylsalicylic acid (6-MSA) and the initiation of sporulation in *P. expansum*, but this required further investigation. However, Sekiguchi and Gaucher (1977) showed that patulin and other metabolites were not a prerequisite for conidiogenesis in *P. urticae*. High concentrations of Ca^{2+} inhibited patulin and griseofulvin production, while stimulating conidiogenesis. Conidia were also produced in mutants blocked at late steps of the patulin biosynthetic pathway. Guzman-de-Pena and Ruiz-Herrera (1997) showed that diaminobutanone, which competitively inhibited ornithine decarboxylase, repressed spore germination. If added after spore germination occurred, it completely blocked both sporulation and aflatoxin biosynthesis. Bennett (1983) reviewed both secondary metabolism

and sporulation, noting that sporulation, like secondary metabolism, was usually induced by a decrease in available nutrients. Both processes start after active growth has stopped and tend to have narrower ranges of trace metal, pH and temperature requirements than growth. Both also occur in distinct families of morphology (differentiation) and chemistry (secondary metabolism). Although analogous, these two processes are often viewed separately and not as part of a single differentiation process.

The nature of the growth medium will dictate the growth form, which in turn dictates the growth kinetics of a fungal culture. Liquid culture provides a more homogeneous environment than solid medium since nutrients are available through diffusion (Martin and Demain, 1978). O_2 is the exception because of its low solubility in water, and can be limiting unless high transfer levels are maintained. The implications of agitation and O_2 transfer have been discussed in Chapter 1. Grootwassink and Gaucher (1980) discuss the virtues of using homogeneous shake cultures over differentiated static cultures, and the ability to separate growth and secondary metabolite phases in fungi clearly using the former.

Much of the focus of research into secondary metabolism has centred on the discovery, identification and production of biologically-active compounds, and therefore the initial research had an organic chemistry emphasis where not only were compounds identified, but also syntheses attempted to parallel the capabilities of producer organisms (which proved difficult because of the structural complexity which exists in many of these compounds). The focus then turned to how the organism produced a secondary metabolite from specific precursors which supplied carbon, hydrogen, oxygen and nitrogen to the compound in question. As a result of such research three main groups of secondary metabolites were determined: polyketides (derived from repeating units of acetate), terpene derivatives (via the isoprenoid pathways), and amino acid derivatives (for example from shikimic acid pathway amino acids). (Campbell, 1983)

2.2 MAJOR PATHWAYS OF SECONDARY METABOLISM.

(from Garraway and Evans, 1984; Bu'Lock, 1961)

2.2.1 Isoprenoids.

Terpenes and sterols are two main products of this pathway which involves the head-to-tail condensation of isoprenoid units. These isoprenes are formed via the mevalonic acid pathway which originates with acetyl CoA. Acetyl CoA is converted to HMG CoA (hydroxymethyl glutaryl CoA) which is in turn converted to mevalonic acid with the eventual production of IPP (isopentenyl phosphate) and DMAP (dimethylallyl phosphate), which are the molecules which undergo condensation into larger units. Examples of these include ergosterol, a ubiquitous fungal steroid, carotenoids, and gibberellins which are plant growth regulators. In terms of the variety of structures the isoprenoids are less important in fungi than in plants in which terpenes are the most abundant natural products.

2.2.2 Polyketides.

This class of compounds is characteristic of fungi, in particular the Deuteromycetes. Acetyl CoA and repeating units of Malonyl CoA are condensed (with the loss of CO_2) to form chains of two carbon precursors (e.g. decaketide = 10 C_2 units), which then cyclise and undergo further reactions such as oxidation, reduction, substitution, ring opening, ring coupling and rearrangements to form polyketide products, such as patulin and the aflatoxins. The process by which these are produced is very similar to that used to make fatty acids but lacks the systematic dehydration and reduction reactions of fatty acid synthesis.

2.2.3 Shikimate-Chorismate.

This pathway produces the aromatic amino acids which can then be converted to secondary metabolites such as cinnamic acid, protocatechuic acid, indoles such as echinulin, ergosterine and lysergic acid through reaction with other compounds such as terpenoids. Phosphoenol pyruvate (PEP) (from glycolysis) and erythrose-4-P (from the pentose phosphate shunt) react together to form dehydroquinic acid which is converted to shikimate, then chorismic acid. This leads into the production of the three aromatic amino acids phenylalanine, tyrosine and tryptophan. This pathway is responsible for the production of many compounds based upon the phenylpropane structure which forms the biogenetical unit of a wide range of plant products, like flavonoids for example. It is also of less importance in the microorganisms.

2.2.4 Non-Aromatic Amino Acids.

Examples of this type of biosynthesis include the β-lactams such as penicillins and cephalosporins. These compounds are formed initially from the condensation of the three amino acids, valine, cysteine and α-amino adipate. Other examples of this type of biosynthesis includes the non-ribosomal production of gramicidin S, a cyclic peptide produced by *Bacillus* species; other cyclic peptides such as phallotoxins; and the production of muscarine from glucose by *Amanita muscaria*. A large number of antibiotics are produced from amino acids and peptides. Some other examples of fungal metabolites include diketopiperazines, gliotoxin and *Amanita* toxins.

2.2.5 Other Forms of Secondary Metabolites.

Secondary metabolites consist of other structural groupings. There are simple acid derivatives, which are a number of simple aliphatic acids not directly related to lipids. Examples include accumulating intermediates of general metabolism such as citric, fumaric and oxalic acids; and modifications or derivatives of general metabolites such as itaconic acid (produced from *cis*-aconitate by a decarboxylase reaction).

Fatty acid derivatives make up another structural group. These include the polyacetylenes of Basidiomycetes, polyene chains, branched chain analogues, propionate-derived C_3 analogues, and the aglycones of macrolides such as erythromycin and polyene antibiotics like lagosin.

Derivatives of sugars form another biosynthetic category. These include simple sugar acids and alcohols that are accumulated by microorganisms (eg. kojic acid derived from glucose); neomycins derived from ribose; and unusual sugars such as amino sugars (desosamine in found in several macrolides), and branched chain sugars (cordycepose in cordycepin) are frequently produced.

Purine and pyrimidine derivatives are also found in secondary metabolism, although little is known about the role of these nitrogen compounds in secondary metabolism. Finally, many compounds are of mixed origin, synthesised from 2 or more of the classes of compounds outlined above.

2.3 ENZYMES OF SECONDARY METABOLISM.

Differences have been observed between the properties of the enzymes of primary and secondary metabolism. Secondary enzymes tend to have a broader substrate specificity and are therefore less sensitive to structural variations in the substrate. This has been exploited in precursor-directed biosynthesis (of penicillins for example) where altered substrates are fed to a producer organism or system resulting in unique structural features which may improve the pharmacological properties. The enzymes of secondary metabolism are also not as efficient as those for primary metabolism since the intracellular concentrations are low and the reactions are catalysed relatively slowly. One example of this is *m*-hydroxybenzyl alcohol dehydrogenase of the patulin biosynthetic pathway in *Penicillium urticae* for which the reverse reaction is thermodynamically favoured (Forrester and Gaucher, 1972b). The enzymes of secondary metabolism form a branching network of alternative pathways, to which acetyl CoA is an important precursor. This compound is the branch-point to terpenes, steroids, fatty acids and their metabolites, and polyketides. Other pathways such as the aromatic amino acids originate from glycolytic intermediates. A more thorough review of regulation of secondary metabolism is given in Section 2.5.

Analogies can be drawn in the nature of reactions of secondary metabolism, despite the diversity of products. Many secondary metabolic pathways involve polymerisation processes, two examples being firstly, the production of peptides from amino acids by synthases involved in non-ribosomal peptide synthesis, and secondly, the condensation of acetyl units (from malonyl units) leading to the synthesis of polyketides, macrolides and polyethers. The acyl and amino acid units for these polymerisations are also both bound to the enzyme/synthase as thioesters with at least 2 types of sulphydryl groups on the enzyme, for example there is a peripheral and a central sulphydryl on 6-MSA synthase. (Behal, 1986)

The enzyme complexes involved in secondary biosynthesis (the synthases involved in the first step of the pathways) dissociate into subunits which are functional although the activity of the complex disappears. These complexes are important to the cell since they eliminate the necessity for intermediates to diffuse from one enzyme to another, which would be limiting to the rate of process. These complexes have been found in subcellular particles but this may be an artifact of the methods used to remove them, and this has been shown by careful

disintegration of cells. The position of secondary enzymes in cells may be important to the resistance mechanisms of the microorganism to its own products, and therefore it would be expected that these would be compartmented. (Behal, 1986)

Although it had been assumed that the limiting factor in secondary metabolite production was the availability of building units in the cells, studies have shown that secondary metabolism is dependent on the activity of secondary enzymes in the cells (Behal, 1986).

2.4 SECONDARY METABOLISM GENE EXPRESSION AND ORGANISATION.

The genes for secondary metabolism in bacteria may be found on the chromosome or on plasmids, and in eukayotic organisms on different chromosomes. Clusters of biosynthetic genes have been found, and linkages commonly occur between the genes for antibiotic resistance and biosynthesis. Sub-clustering, for example in genes for the production of tetraenomycin and actinorhodin, may act as a control mechanism for the timing and sequential appearance of each enzyme in the pathway. This is achieved in prokaryotes by coupling transcription and translation so that the product of the preceding enzyme may be acted upon. The sequential formation of enzymes in fungi has been shown, however the most efficient means by which to ensure the spatial constraints for sequential reactions to occur is through the formation of multi-catalytic site proteins or multienzyme complexes, onto which the intermediates are bound, ensuring their availability for reaction. In eukaryotic organisms, compartmentation of enzymes can also ensure the spatial availability of reaction products to the appropriate enzymes.

Coordination of the expression of biosynthetic and resistance genes is necessary in order to avoid suicide of the producer organism, and therefore induction and repression mechanisms are involved. In organisms such as *S. griseus* and *S. rimosus*, *tetA* and *tetB* resistance genes are induced by tetracycline, the toxic end product. For the production of streptomycin by *S. griseus*, positive and negative controls effect the production of the SPH protein. SPH is induced by streptomycin (the final product). An open reading frame (ORF downstream of *strA* is required for induction, and is likely to encode a repressor protein. The *strR* gene

positively regulates the *strA* gene also. Two or more resistance mechanisms can be found in some *Streptomyces* spp. with at least one linked to the cluster of biosynthetic genes. Another case of resistance regulation is where the resistance gene catalyses a reaction in the biosynthetic pathway, so that the resistance and biosynthetic genes are under the same controlling mechanism. A-factor of *Streptomyces* is involved in the induction of resistance as well as biosynthesis.

Some bacteria (*E. coli* and *B. subtilis*) have been shown to utilise a mechanism involving the production of alternative sigma factors or ancilliary proteins that are able to alter the specificity of RNA polymerase. Promoters for antibiotic resistance and biosynthesis genes have been cloned, and show no resemblance to the normal consensus promoters sequences. It is possible that most secondary metabolism gene promoters have a peculiar structure and are therefore only able to be expressed under specific conditions leading to secondary metabolism. Evidence has shown that multiple sigma factors exist, but the physiological significance of different RNA polymerases under different conditions is not clear. Ancilliary regulatory proteins may also play an important role in controlling differential gene expression in secondary biosynthesis. A model has been described for the *spoA* dependent inactivation of the *B. subtilis* gene *abrB*, which is an ancilliary protein proposed to be a negative regulator of *tycA* transcription (a secondary metabolism gene with a promoter resembling the canonical structure of the main class of promoters found in this species). The nutritionally regulated inactivation of AbrB would result in the late induction of *tycA* synthesis in secondary metabolism. (Martin and Liras, 1989).

2.5 REGULATORY MECHANISMS.

The production of secondary metabolites is delayed until idiophase by a variety of mechanisms; this is advantageous to antibiotic producers that are sensitive to their product during growth phase. This also allows for an advantage in nutritionally poor conditions (under which secondary metabolites are produced) for the antibiotic/toxin producer to potentially compete with other microorganisms.

2.5.1 Enzyme Repression and Inhibition. (Martin and Demain, 1980)

The control of initiation of secondary metabolite synthesis can be controlled by repression (prevention of gene transcription) or inhibition of biosynthetic enzymes. Since expression of biosynthesis does not occur at high growth rates it can be assumed that the synthases are not formed or are inhibited. The nature of regulation does not have to be exerted at a single level and it is possible to find both inhibition and repression involved. For example the onset of biosynthesis could be repressed until a specific nutrient is exhausted, while synthesis of compounds could be inhibited by a controlling factor until after the synthase is formed. Late enzyme formation has been noted in the production of some antibiotics, therefore control occurred possibly by interference with gene transcription. An example of this is amidino-transferase and streptidine kinase in streptomycin biosynthesis, which are both repressed during the growth phase. The amidinotransferase is synthesised *de novo* since the process can be inhibited by chloramphenicol.

The delay of the onset of secondary metabolite biosynthesis can also be due to inhibition of preexisting enzymes, unable to operate because of the presence of an inhibitor. β-Lactam antibiotic production by *Cephalosporium acremonium* occurs after the growth phase has ended, but is not inhibited by protein synthesis inhibitors.

Feedback inhibition (also called end-product or allosteric inhibition) allows a cell to monitor the production of a particular compound so that its synthesis may be switched off when a certain level is reached, or switched on if concentrations drop too low. The final metabolite of a biochemical sequence inhibits the action of an enzyme (usually the first) of that sequence, by binding to it at a site separate to the substrate binding site so that the attachment of the substrate to this is impaired. Repression occurs when a derivative of the end product, possibly the product in combination with an aporepressor such as a protein, inhibits the formation of enzymes (or their mRNA) in a pathway, to control the capacity for synthesis of certain proteins (Demain, 1966 and 1968). The synthesis-inhibiting level in a producer strain is often similar to the production level. Mycophenolic acid causes 68% inhibition of SAM:demethyl-mycophenolic acid-*O*-methyltransferase, the final step in its synthesis (Martin and Demain, 1980).

Inhibition can also occur in a pathway where there is a common branch between primary metabolite formation and secondary metabolism, where the primary end product feeds back to inhibit the common part of the pathway. One example of this is the inhibition of penicillin production in *P. chrysogenum* by lysine (Martin and Demain, 1980).

Most end products are formed from branched pathways, and therefore there have been three mechanisms identified for the prevention of interference by one end product on another derived from the same metabolic pathway, so that the excess of one does not impact negatively on the others (Demain, 1966). The production of multiple enzymes or isoenzymes is one means by which this can be achieved. Each isozyme catalyses the same reaction but is controlled by a different end product. Multivalent (or concerted) feedback requires all end products to be present in excess in order for repression or inhibition to occur. Finally, cumulative feedback occurs when each end product can provide only limited inhibition singularly, with increases in inhibitory effects when combinations of end products are involved. Complete inhibition requires all the end products to be present.

2.5.2 Mechanisms to control initiation of biosynthesis.

Two models describe the possible mechanisms for controlling the start of secondary metabolism. The first involves a small molecule which acts as a corepressor or inhibitor which inhibits/ represses synthases, and which must be depleted in order for synthesis to occur. In the second, an inducer or an activator must be synthesised to initiate biosynthesis.

Carbon catabolite repression is the repression of synthesis of enzymes by the catabolic products of a rapidly assimilated carbon source (Demain, 1968) which is often glucose. For many fermentations oligo- or polysaccharides are better carbon sources than monosaccharides, and it is necessary to add a more slowly-utilised carbon source to a medium containing glucose. The glucose is used first in the absence of any production, followed by the second carbon source for antibiotic/secondary metabolite synthesis. Other carbon sources can also be involved, for example citrate suppresses novobiocin production, but production will occur when glucose is used in the secondary phase. Since many of the preferred carbon sources are metabolised through the same intermediates as glucose, it would appear that a precursor effect is not operating.

A molecular mechanism may be related to the growth rate control of secondary metabolism. Slow carbon feed results in slow growth rate therefore eliminating interference by glucose or similar. In a few cases glucose has been shown to repress a known biosynthetic enzyme, two examples being phenoxazinone synthase (PHS) of actinomycin biosynthesis, the enzyme forming the phenoxazinone nucleus of actinomycin. PHS mRNA levels increased only after all the glucose in the medium was exhausted (Martin and Liras, 1989). A phosphatase in neomycin biosynthesis has been shown to be under glucose repression also.

Other mechanisms have been proposed, and these include decreases in pH and depletion of dissolved O_2. Lowering of pH can come about through the accumulation of undissociated forms of pyruvic and acetic acid due to high glucose concentrations. pH regulation of gene expression in *A. nidulans* has been studied and shown to be mediated by a Zn finger TF PacC, which operates with 6 genes involved in a pH signal transduction pathway. Full length PacC (73 kDa) is converted to a functional proteolysed form (29 kDa) under alkaline conditions. Mutation disrupting the *pal A, B, C, F, H* and *I* genes prevent this conversion and results in effects reflecting growth at acidic pHs (Negrete-Urtasun *et al.*, 1997). cAMP has been considered a possible effector molecule for catabolite repression in some microorganisms in which it acts as a positive effector of catabolite repression of inducible catabolic enzymes, since high glucose concentrations inhibit adenylate cyclase (which produces cAMP), therefore lowering intracellular concentrations. cAMP binds to a receptor protein to form a complex which can then interact at promoters for inducible enzymes. It is believed that cAMP is more closely related to phosphate regulation than carbon regulation, and possibly inhibitory to synthases in high levels (Martin and Demain, 1980).

Regulation of secondary metabolism by nitrogen metabolites has been reported in many cases. Ammonium (NH_4^+) is a repressor of enzymes involved in the utilisation of other N sources including nitrite and nitrate reductases, NAD-dependent glutamate dehydrogenase, and arginase. In fungi an enzyme protein appears to be involved in N metabolite regulation, requiring NH_4^+, α-ketoglutarate, and a catalytically active NAD specific glutamate dehydrogenase for repression to occur. Media for antibiotic production frequently utilise slowly metabolised amino acids resulting in N limitation. An example of this is the use of proline as the N source for the streptomycin fermentation. The production of patulin by *P. urticae* was delayed by the presence of NH_4^+ in the fermentation medium, especially if added

during the 7 hour period of derepression leading to the formation of secondary enzymes and idiophase proteinases. Secondary enzyme production was more sensitive to this type of regulation than the proteinases (Rollins and Gaucher, 1994). The appearance of the first enzyme of the patulin pathway occurred at an NH_4^+) concentration of 2.5 mM. These authors consider the case of primary nitrogen repression mechanisms in other fungi in explanation of what may have been occurring in *P. urticae.*

The general derepression of genes regulated by nitrogen (for example the genes for nitrate or amino acid utilisation) operates through the action of a DNA-binding regulatory protein in response to the depletion of the preferred nitrogen source. The control of derepression is mediated through 2 signals-a global regulatory gene product and a pathway specific gene product. The global regulatory genes are *trans*-acting positive effectors belonging to the GATA family of transcription factors, which contain a central GATA core sequence and bind DNA by a cys_2/cys_2-type zinc finger motif. These include AREA (*areA*) from *Aspergillus nidulans*, NIT2 (*nit-2*) from *Neurospora crassa*, and NRE (*nre*) from *Penicillium chrysogenum*, which have a significant degree of sequence identity to each other, especially in the 50 residue region involved in DNA binding.

These proteins may bind other proteins or glutamine (the critical metabolite exerting N metabolite repression) at the carboxy terminus. The pathway specific regulatory factors are believed to be activated through binding a specific inducer, and many belong to the GAL4 family of regulatory proteins, which contain a cys_6/Zn_2-type binuclear Zn cluster (unique to fungi) for DNA binding. These proteins interact/cooperate with the global-acting regulators to switch on specific sets of N catabolic genes, depending on substrate availability and N requirements. An example is NIT2, which has been shown to bind to NIT4 (through its Zn finger) to regulate expression of the nitrate reductase (*nit-3*) gene in *N. crassa.* As mentioned, glutamine is responsible for the specific induction of N derepression. It is believed to operate either unchanged or as a derivative compound, possibly through recognition by a global regulator-pathway specific regulator complex, or by binding individually to the global regulator or the pathway regulator. The nature of the signal transduction pathway which senses repressing levels of glutamine, or whether or not an unidentified factor is involved in glutamine detection (to transfer the signal to the global proteins) are unknown. (Marzluf, 1997)

Evans and Ratledge (1985) studied the roles of several enzymes in lipid accumulation by the oleaginous yeast *Rhodosporidium toruloides* CBS 14. They proposed that AMP deaminase activity was higher in nitrogen-limited cells irrespective of the N source. The exhaustion of cAMP immediately after N depletion in another lipid accumulating yeast indicated that adenylate energy charge regulation could be related to lipogenesis. It was proposed that AMP deaminase could quickly create a low intracellular AMP concentration, leading to a rapid increase of citrate accumulation through inhibition of NAD-ICDH. The NH_4^+ produced by the deaminase reaction could then stimulate glycolysis since it is a positive effector of phosphofructokinase and pyruvate kinase. It also stimulates ATP:citrate lyase activity, which generates acetyl CoA, leading to lipid formation.

Kim *et al.* (1995) showed that the intracellular ammonium level of a citric acid over-producer *A. niger* could be controlled by specific O_2 uptake rate. NH_4^+ has been shown to strongly regulate the glycolytic enzyme phosphofructokinase, and may counteract the feedback inhibition of citrate on this enzyme, therefore allowing citrate overproduction to occur. The specific oxygen uptake rate and intracellular ammonium ion concentration were shown to be closely correlated through pulsed feeding of ammonium to fed-batch cultures.

The production of water-soluble red pigments by *Monascus* sp. was shown to be reliant on ammonium salts as the N source, but appeared to be repressed when NH_4NO_3 was used as the sole N source in the fermentations. Experiments revealed that in this case, the NH_4NO_3 was not involved in a N repression process, or in the enhancement of synthase decay. Instead, the observed effects were due to the poor ability of NH_4NO_3 to donate N to the Schiff-base reaction in the production of red pigments from orange ones (Lin and Demain, 1995).

Doull and Vining (1990) showed that N catabolite repression was probably operating in *Streptomyces coelicolor* A3(2) production of actinorhodin, which occurs via a polyketide intermediate. N limitation resulted in a higher rate of antibiotic synthesis. Another polyketide-producing member of this genus, *S. ambofaciens*, was shown to undergo regulation depending on the N source. In the production of spiramycin, the formation of malonyl CoA, the activated intermediate, is proposed to occur via two pathways. Firstly, oxaloacetate dehydrogenase was shown to be induced (or possibly derepressed), after growth on NH_4^+,

during idiophase; and secondly, acetyl CoA carboxylase was induced, after growth on valine, during idiophase (Laakel *et al.*, 1994).

Beyond induction/derepression type mechanisms, other factors may affect how N regulates secondary metabolism. The transport of NH_4^+ will affect the its pool size and therefore any further interaction it has with secondary metabolism. The intracellular concentration of NH_4^+ was shown to regulate the synthesis of some of the protein components of its uptake system by inhibition and repression in *A. nidulans*. In the fungus *Stemphylum botrysum*, the NH_4^+ ammonium transport system is regulated by L-amides. (Aharonowitz, 1980). In fungi, amino acid transport is also affected by NH_4^+, which inhibits transport due to its uptake into cells. This was demonstrated in *S. cerevisiae*, in which the NH_4^+ was incorporated into α-amino acids such as glutamate, demonstarting that the enzymic activity of NADP-glutamate dehydrogenase (anabolic) inhibits amino acid transport, rather than its possible function as a regulatory element (Aharonowitz, 1980). Intracellular amino acid pools alter during the growth cycle, and are affected considerably by environmental factors such as NaCl concentration. High amino acid accumulations only occur during stationary or slow periods of growth, and it is assumed that the rate of amino acid uptake corresponds with the rate of macromolecular synthesis.

The relationship between aromatic amino acid biosynthesis and secondary metabolism in higher plants was examined by Jensen *et al.* (1989) and provides another perspective for regulation of metabolism by N compounds. Aromatic amino acid biosynthesis was traditionally regarded as being restricted to chloroplasts, however, evidence has been presented to support the hypothesis than it could occur in the cytosol as well, catalysed by different isozymes of the enzymes involved in the chloroplast pathway. Aromatic amino acid biosynthesis in the cytosol would interface directly with the secondary processes which occur at this location, and could be regulated or manipulated differently to the chloroplast pathway although some interaction between the intracellular pools may occur.

Phosphate is a crucial growth-limiting nutrient in many fermentations belonging to different biosynthetic groups including peptides, polyene macrolides, and tetracyclines. High phosphate concentrations repress the formation of several antibiotics, including *p*-amino benzoic acid synthase (*pabS* gene) in candicidin synthesis. *pabS* mRNA decreased by 95%

when high levels of P were added. The effect was specific to this gene since the total mRNA synthesis was enhanced under these conditions. A P-regulated promoter upstream of the *pabS* structural gene was subsequently cloned (Martin and Liras, 1989). Studies have been done to ascertain whether or not intracellular orthophosphate is the ultimate effector molecule, or if it regulates another intracellular effector to control biosynthetic expression. ATP levels increased after phosphate addition to *Streptomyces griseus*, this occurred just prior to inhibition of antibiotic synthesis. The rate of protein production did not change, therefore raising the possibility ATP could act as an intracellular effector. The adenylate energy charge hypothesis of Atkinson and Watson (1967; See Chapter 1.1.4) is a linear measure of the amount of energy stored at any time in the adenylate system, and was proposed to be a regulatory parameter coordinating energy-utilising and energy-generating metabolic pathways, however according to Demain and Martin (1980), this is a somewhat controversial view.

A variety of mechanisms have been suggested for control of secondary metabolism by inorganic phosphate (P_i), however most have been disproven or lack sufficient evidence. One possible inhibitory site for regulation is alkaline phosphatase, whose activity and synthesis is suppressed by P_i. Concentrations lower than those supporting the vegetative growth of plant microbial or animal cells were needed for secondary metabolism to occur. Alkaline phosphatase is required for the synthesis of many secondary metabolites, and is derepressed in low P_i concentrations. Phosphatases are often formed prior to the production of active antibiotics such as streptomycin, viomycin and vancomycin, and it has been suggested that high concentrations of P_i prevent the synthesis of such antibiotics by repressing these enzymes (Weinberg, 1974). A phosphate-regulated promoter sequence for *pab* genes involved in candicidin synthesis has been determined in *S. griseus*, an organism which has been shown to undergo P_i repression (Doull and Vining, 1990).

With respect to phosphate regulation some biosynthetic intermediates are phophorylated whereas end products are not. Generally, microbial phosphatases that cleave phosphorylated intermediates are often regulated via feedback inhibition or repression by inorganic phosphate.

Induction refers to enzymes which are normally absent or barely detectable but which are formed rapidly after the addition of substrate or substrate analogues, in a process that is the reversal of repression (which is also a description of derepression) (Demain, 1968). It is

sometimes difficult to determine whether the stimulatory effect is true induction or a precursor effect. Inducers include those compounds which stimulate biosynthesis when added to growth phase (before idiophase), but not when added to idiophase in which protein synthesis is inhibited by the addition of an inhibitor; and which can be replaced by a non-precursor analogue. By contrast, precursors stimulate synthesis when added during idiophase even when protein synthesis has been blocked. Initiator accumulation may cause two courses of events: a preexisting enzyme which was only slightly active because of low substrate levels or other competing reactions now becomes functional; or the initiator induces (or derepresses) the formation of enzymes responsible for product formation. The closure of the usual biosynthetic and energy generating cyclic pathways at the end of tropophase results in an accumulation in the concentrations of initiators, and could result in the induction of secondary metabolic pathways (Demain, 1968).

A-factor (2-*S*-isocaprolyl-3-*R*-hydroxymethyl-γ-butyrolactone) is a pleiotropic effector responsible for the stimulation of streptomycin production and resistance, and sporulation in *S. griseus* (Martin and Liras, 1989). Its effects are most marked if added at the time of inoculation rather than later (after 48h), even in brief treatments of 3-4 minutes. Although the molecular mechanism of A-factor is unknown, it is reported to have an effect on carbohydrate metabolism, and control occurs at the transcriptional level. Glucose-6-phosphate dehydrogenase (G6PDH) activity is higher in non-antibiotic-producing mutants and almost undetectable in high-producing mutant strains. Upon addition of A-factor G6PDH activity decreases immediately although this does not affect the pure isolated enzyme, therefore another compound may be formed in response to A-factor to mediate this effect. One candidate is a breakdown product of NADP called adenosine diphosphoribose, which can selectively inhibit G6PDH activity, which possibly leads to the utilisation of glucose through alternative routes resulting in streptomycin production. In *N. crassa* this compound influenced sporulation, and a role for its involvement in this same process in *S. griseus* has been demonstrated (Khokhlov and Tovakova, 1979). A-factor genes have been cloned from various *Streptomyces* spp., *afsA* from *S. bikiniensis* (A-factor), *afsB* from *S. coelicolor.* The products from these genes are suitably similar that they may be transferred into A-factor deficient mutants to successfully induce secondary metabolism. A cascade of expression is believed to be initiated by genes such as *afsB* to control secondary metabolism and differentiation in *Streptomyces* spp. Another gene, *saf* (secondary metabolism activating

factor), was cloned from *S. griseus*, and includes a DNA-binding domain in it structure. This acts as a common control factor for at least 5 extracellular enzymes, pigment formation, and differentiation. It is believed that *saf* may interact directly with regulatory sequences of secondary metabolism, or have an involvement in A-factor formation, which in turn controls activation of gene clusters for secondary metabolism (Martin and Liras, 1989).

2.5.3 Mechanisms to terminate secondary biosynthesis.

Variations in the duration of secondary metabolite biosynthesis range from 4 hours through to several days providing non-repressive or non-inhibitory conditions are provided, and varies between strains and environmental conditions. Feedback regulation has already been examined (Section 2.5.1), but forms a major means for the cessation of synthesis to occur. Irreversible decay of one or more of the enzymes of the biosynthetic pathway is another way this can be achieved. Natural immobilisation of the enzyme complex can occur as in the case of the soluble bacitracin synthase of *Bacillus licheniformis* which becomes cell bound later in growth phase. This allows protection against the intracellular proteases which become active at this time. Gramicidin S synthase is also inactivated by O_2 in a similar process independent of proteases. Intracellular proteinase activity is reponsible for the degradation of patulin pathway activity in *P.urticae*, and is controlled by the ammonium level in a manner similar to the control of the initiation of biosynthesis (Rollins and Gaucher, 1994). A third means for the cessation of secondary metabolite synthesis, but for which there is no experimental evidence, is the depletion of intermediary precursors of the final metabolite. (Martin and Demain, 1980)

2.5.4 Control Effects by Environmental Factors.

Inorganic compounds such as phosphate can affect the production of secondary metabolites even though they are not incorporated into the product. Inorganic phosphate concentration is usually critical with respect to avoiding excess concentrations. Among the effects observed are increased rates of sugar utilisation, mycelial nitrogen content, RNA and DNA and the transient concentration of pyruvate excreted into the medium. Some pentose phosphate enzymes have been shown to be inhibited by phosphate, shifting sugar metabolism to the Embden-Meyerhof-Parnas pathway. Manganese (Mn^{2+}) is reported to be a stimulant of secondary metabolite production in *Bacillus*, unable to be replaced by other elements, but able to be blocked by mRNA and protein synthesis inhibitors, indicating that it could be related to

some sort of derepression control mechanism. Manganese plays a major role in the regulation of manganese peroxidase (MnP) expression, involved in lignin degradation in white rot fungi such as *Phaenerochaete chrysosporium* and *Dichomitus squalens* (Perie and Gold, 1991; Brown *et al.*, 1990). Brown *et al.* (1990) showed that the enzyme was only detectable in cells grown in the presence of Mn, and that its induction was blocked in the presence of transcriptional inhibitors, indicating a role for Mn as a transcriptional regulator as well as the substrate. The regulation of MnP and lignin peroxidase is believed to be mediated by cAMP since changes in intracellular levels of this compound resulted in differential regulation of these two enzymes (Boominathan and Reddy, 1992). The lack of LiP activity in cultures with high concentrations of Mn could possibly be due to increased levels of phosphodiesterase (stimulated by Mn) which degrades cAMP.

Scott *et al.* (1984) showed that Mn^{2+} operated at the gene level to induce the patulin biosynthetic pathway beyond the first committed metabolite, 6-MSA in *P. urticae*. In Mn-supplemented cultures patulin production prevented the accumulation of 6-MSA seen in Mn-deficient cultures, in which there was minimal production of patulin (Scott *et al.*, 1986). *m*-hydroxybenzylalcohol dehydrogenase (a patulin biosynthetic pathway enzyme beyond 6-MSA) activities were significantly lower in Mn deficient cultures, and a dose-response relationship was established between patulin production and Mn concentration.

O_2 transfer is another environmental factor which can have critical effects on metabolism (as discussed in Chapter 1), and is normally supplied in fermentations to support biosynthesis. In some cases an oversupply of O_2 can be inhibitory to product formation, and therefore may be involved in regulation of metabolism in some way. O_2 is involved in glucose catabolite repression (which can be overcome by anaerobic shock), and is responsible for diverting glucose metabolism through the pentose phosphate pathway away from the EMP pathway. The production of a range of metabolites is affected by O_2, and includes geosmin, a sesquiterpenoid which is produced by *P. expansum* (believed to be effected through mixed function oxidases – MFOs - which may be involved in the biosynthesis of geosmin). This was stimulated under increased O_2 atmospheres (Dionigi and Ingram, 1994). On the other hand, red pigment production by *Monascus purpureus* was inhibited under increased aeration conditions, possibly since the production of this pigment requires ethanol as a carbon source, which is maintained under the fermentative growth conditions low O_2 provides.

Lipids have proven beneficial to fermentations through the inhibition of key enzymes of lipid synthesis, TCA cycle and the HMP pathway (acetyl CoA carboxylase, citrate synthase, and glucose-6-phosphate dehydrogenase) by CoA thioesters of fatty acids, therefore preventing flux into undesirable pathways. Lipids have also been used as major carbon sources, replacing carbohydrates in fermentations which do not utilise polyketide biosyntheses (which would be feedback-inhibited under these conditions).

Temperature has also been shown to play a role in affecting fermentation processes, with different temperature optima being required for tropophase and idiophase. Idiophase temperature optima tend to be below that for normal growth, for example citric acid production by *A. niger* was optimal at 26.5°C despite an optimum for growth being 37°C. Basic control mechanisms can be sensitive to temperature manipulation, the end-product inhibition of initial enzymes of amino acid pathways occur at lower temperatures than that for normal growth so that the lowering of temperature after tropophase in a fermentation leads to a greater shutting-off of these normal pathways encouraging further the transfer to idiophase metabolism. (Demain, 1968).

In studies with Basidiomycetes, secondary metabolite production has been induced by toxic secondary metabolites from other fungi of the same class, causing retardation of growth and extensive stimulation of synthesis. The process was unrelated to the presence of cell wall materials like in fungal/plant interactions, and occurred before any cell contact had occurred (Sonnenbichler *et al.*, 1994). For example Fomanosin and fomanoxin from *Heterobasidion annosum* were response-inducing metabolites.

In many industrial fermentation processes it is important to overcome some of these mechanisms in order to achieve overproduction of the desired metabolite by adding inducers, removal of repressors, and the genetic manipulation of the enzyme forming systems to reduce the effect of regulation.

Mutation is one means by which this can be achieved, with the aim of producing mutants resistant to catabolite repression, constitutive mutants which are able to produce the desired enzyme in the presence of a repressor, or which are able to form enzymes without an inducer

being present. The reversion technique produces mutants which often have higher production capabilities than the wild type grandparent. Reverse auxotrophic mutations or reversion can be used to excrete end products of derepressed enzyme systems or desensitised enzymes. Reversions in structural genes can result in enzymes which differ structurally from the original enzyme but are still active, however it differs from the original because it is insensitive to feedback regulation (Demain, 1966). Reversion can affect many genetic characteristics of the culture other than the intended locus, resulting in pleiotropic mutants from the reverse mutation which is useful for selection (Demain, 1968).

Breaking feedback regulation can be achieved in two ways. The first, and simplest, is to decrease the concentration of the end product, an example of this is the fermentation to produce ornithine. Ornithine is an intermediate on the arginine pathway, which is unbranched. By using an organism (*Corynebacterium glutamicum*) mutated in the enzyme catalysing the step after ornithine, and feeding low levels of the end product, arginine, to overcome inhibition of an earlier enzyme on the pathway, high levels of ornithine are excreted into the medium (Demain, 1966 and 1968).

If the end product of a branched pathway is the product of interest the removal of feedback regulation is easier. The reversal of feedback inhibiton can be overcome by the other end products of the same pathway. For example, lysine is produced from a pathway which also results in threonine production, these branch from each other after the second step of the pathway. Removal of the enzyme on the threonine branch, in a *C. glutamicum* mutant, regulates the intracellular concentration of threonine, which inhibits the first enzyme of the pathway. In lysine producing mutants a mixture of lysine and threonine inhibits this first step by multivalent (or concerted) feedback inhibition. As a result, low threonine concentrations causes a high level of lysine production.

A second way to overcome feedback regulation is alteration of enzymes or enzyme forming mechanisms. This is relevant to unbranched pathways where limiting the concentration of an end product is not desirable and requires modification of an enzyme so it is less sensitive to end-product inhibition, or alteration of the enzyme-forming system so it is less prone to end-product inhibition. One way to achieve this is to isolate mutants resistant to growth inhibitory analogues of the desired end product, so that the analogue mimics the natural compound in its

feedback function and since this analogues is unable to be used for macromolecular synthesis growth is inhibited. In this way the inhibitory analogue selects for rare mutants with altered enzyme or enzyme-forming systems which are insensitive to feedback regulation, and therefore these mutants can overproduce and excrete the end product. For example, trifluoroleucine-resistant mutants of *Salmonella typhimurium* excrete leucine as a result of a mutation controlling repression of the leucine pathway (Demain, 1966 and 1968).

CHAPTER 3

GROWTH CONDITIONS FOR THE STUDY OF METABOLISM IN *PENICILLIUM EXPANSUM.*

3.1 INTRODUCTION

Four different growing regimes were employed for this study in order to determine the effects of agitation and nutrient levels on secondary metabolism in *Penicillium expansum.* A nutrient poor medium was provided by the Malt Extract (ME) medium, which contained only malt extract and peptone (Woodhead and Walker, 1975). The nutrient rich medium was the Yeast Extract Glucose Buffer medium (YEGB) as used by Grootwassink and Gaucher (1980), which included yeast extract, glucose, trace metals and a buffer solution.

Agitation on an orbital shaker was used to maintain a high pO_2 in the cultures. Fernbach flasks (1 L) were used for shake cultures because the squat shape gave a greater liquid surface area to allow better aeration. Due to their size and shape only 6 flasks could be placed on the shaker at any one time. This meant that for a four day culture run, the cultures could not be duplicated due to space limitations, unless a second shaker was used, however there was not one available. Therefore the static cultures were conducted in a similar manner for consistency. This would lead to an increased degree of variation within the experiments conducted, since the experimental units in each set were separate from others. This is known statistically as blocking.

This aspect of the problem could have been overcome by using smaller Erlenmeyer flasks which would have allowed a greater number to be used for each run, but these would not have provided as great a degree of agitation as did the Fernbach flasks. Similarly, sub-sampling of one culture throughout the four days would also overcome

any variation in having separate experimental units within a run, however this was considered less practical and a potential source of contamination. Therefore the approach used for the analysis of results was to run several separate experiments for which the parameters could be averaged and the degree of variability reported as the standard error i.e. Std. Error = $\sigma/\sqrt{n}$ where σ is the standard deviation, and n is the number of replicates. The averages and their standard errors for each parameter could then be compared between shake and static cultures. The standard error results have been used as the error bars on graphed results throughout this thesis.

The use of continuous culture was considered for use in this study, however time constraints, and equipment problems, resulted in the main focus being on completeness of the batch culture studies. A chemostat approach had been planned where metabolic parameters (primary enzymes, secondary metabolites, lipids, etc) would be measured at specific dissolved O_2 (DO) levels while the other nutrients and conditions would be maintained at a constant level. This should allow the effects of DO concentration to be distinguished from other associated effects that have been observed in the batch cultures. This work was not undertaken due to technical problems with the apparatus.

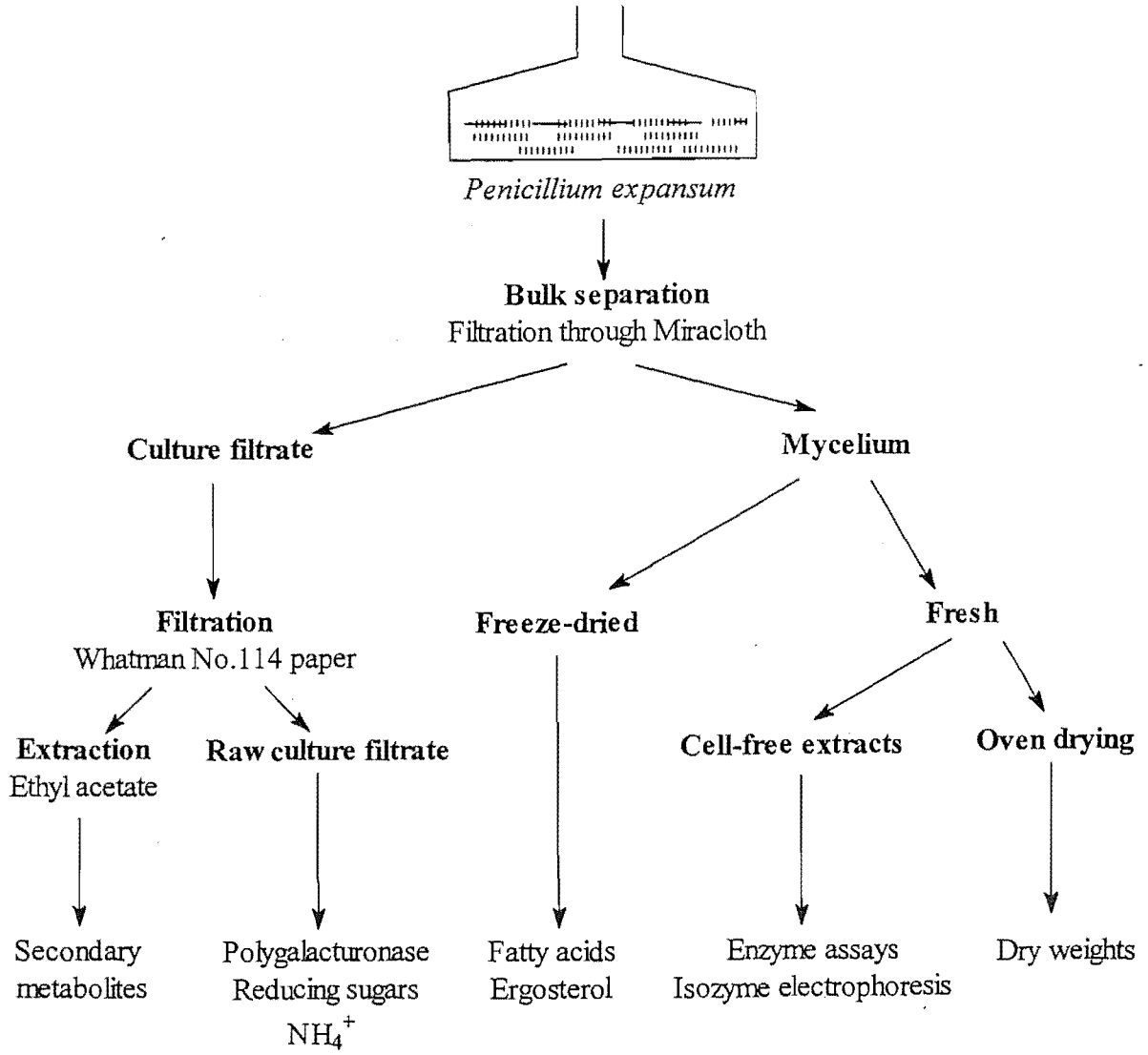
The general objectives of this section of work were:

1 Study of the growth kinetics in YEGB and ME media under shake and static conditions.

2 Monitoring of the morphological changes and differences in the two media under shake and static conditions.

These could then be used in consideration with the results for the metabolic parameters monitored in other sections of work in this thesis.

Figure 2. Overview of culture usage.



3.2 MATERIALS AND METHODS.

3.2.1 Organism.

Penicillium expansum was isolated from mouldy apples collected from a local orchard and compared to a strain (F96) from the PAMS department culture collection, and a strain isolated by Peter Ferrar during his PhD studies. Paper chromatography of ethyl acetate extracts of liquid culture medium (see Chapter 5) was used to determine which strain readily produced patulin based on whichever sample had the most intense patulin spot, given that the method and quantities used in extraction were consistent for each. The strain isolated from the mouldy apples proved to be the best.

3.2.2 Storage.

P. expansum cultures were maintained at 4 °C on potato sucrose agar (PSA) slopes prior to inoculation into culture media; PSA being a better medium for sporulation. These slopes were incubated at 22 °C under strong lighting to stimulate sporulation. Slopes were inoculated from potato dextrose agar (PDA) plates of this organism. For long term storage, plugs of *P. expansum* from solid media were placed in a 10 % v/v glycerol solution and frozen at -20 °C for 1 h, then held at -80 °C until required. These cultures were defrosted slowly to room temperature then plugs placed onto PDA plates containing streptomycin and incubated at 26 °C. This treatment retained the morphological and physiological characteristics (including secondary metabolism) of this fungus, and overcame any problems or changes to the organism that were likely to have arisen through long term storage at 4 °C.

3.2.3 Liquid culture media.

3.2.3.1 2 % Malt Extract Medium (ME); 16g malt extract (Difco) and 3g peptone (Difco) were dissolved in 1 L distilled H_2O, then autoclaved at 121 °C for 15 minutes. Typical analysis of malt extract gives a composition of 1 % w/w total N, 0.6 % w/w amino N, 0.1 % w/w NaCl, pH 5.6.

3.2.3.2 Yeast Extract Glucose Buffer Medium (YEGB).

Solution 1: Yeast Extract 5g/L in final medium volume.

Solution 2: Glucose 40 g/L in final medium volume.

Solution 3: Final concentrations.

13.6 g/L KH_2PO_4, 9.8 g/L citric acid,1 g/L Na_2SO_4,10 mL/L trace metals* solution adjusted to pH 6.3 using NaOH

The three solutions were made up separately in distilled H_2O, autoclaved separately and mixed together prior to inoculation.

*Trace Metals Solution (Yamamoto and Segel, 1966): 100 mL

0.5g	Na citrate	20mg	$CuCl_2$	0.2g	$ZnCl_2$
0.3g	$MnCl_2$	75mg	$CaCl_2$	20mg	$CoCl_2.6H_2O$
5g	$MgCl_2.6H_2O$	0.2g	$FeCl_3$	10mg	Na molybdate

0.2 mL $CHCl_3$ was added to preserve this solution. This was made fresh every 2 months.

3.2.4 Culture conditions.

3.2.4.1 Inoculation procedure.

A 10 mL spore suspension containing 6 x 10^6 spores/mL was prepared by adding a 0.05 % v/v solution of Tween 80 in distilled H_2O to week-old slopes and inverting gently. A calibration curve of absorbance at 600 nm of spore suspensions of varying concentrations (verified by haemocytometer counts) was prepared using a 1:4 dilution of the spore solutions in 0.05 % v/v Tween 80. This resulted in a curve that was linear to 7.5 x 10^6 spores/mL (1/4 dilution), from which the concentrations of the unknown spore preparations could be determined and the appropriate dilutions made to bring the concentrations to 6 x 10^6/ mL. Several repeats of this method resulted in absorbances very close to each other for each concentration of spores chosen, indicating the reproducibility and reliability of this method. 10 mL of the spore suspension was inoculated into 200 mL liquid culture medium to start each culture.

3.2.4.2 Culture vessels and aeration.

Apart from some initial experiments using 250 mL Erlenmeyer flasks, the majority of liquid culture work on primary enzymes was carried out using fungi grown in 1 L

Fernbach flasks containing 200 mL culture medium. An agitation speed of 300 rpm was chosen since this gave adequate agitation of the culture medium.

Static cultures were also grown in 200 mL medium in Fernbach flasks, however due to a shortage of these, 1 L Roux bottles containing 200 mL medium were substituted with no change to the growth or physiological characteristics of the organism observed. All cultures were incubated at 26 °C.

3.2.5 Dry Weight Estimation.

The wet mycelial mat was filtered through Miracloth and weighed before a portion of this was taken and weighed. This portion was then oven-dried overnight at 90 °C and the dry weight noted. The total dry weight was calculated by mutiplying the total wet mycelial weight by the weight of the dry portion divided by the weight of the wet portion. This allowed the remainder of the mycelium to be used for other analyses.

3.3 RESULTS AND DISCUSSION.

3.3.1 Dry Weights.

Figures 3 and 4 show the average dry weights for shake and static cultures in ME and YEGB media respectively. The ME shake cultures exhibited a reasonable rate of growth from the beginning of the culture period and this decreased gradually over 4 days to almost zero at 96 hours. The dry weight levelled off after 72 hours having increased steadily till then. The static ME cultures showed very little growth over the first 24 hours. The growth rate increased gradually to 72 hours and then decreased. There were significantly lower dry weights in the static cultures, the 96 hour cultures reached only about half the dry weights of the shake cultures.

The YE shake cultures started with the highest average growth rate at any time under any of the conditions measured. This decreased over the next two 24 hour periods to 72 hours, falling to zero in the last 24 hour period to 96 hours, when the culture stopped growing. The YE static cultures started with an extremely low growth rate as for the ME cultures, however the next 24 hour period to 48 hours was marked by a sharp increase in growth rate to higher than that of the shake cultures at the same time. This growth rate was maintained till 72 hours, after which time there was a decrease. The YE static cultures differed from the ME cultures because the former accumulated biomass to reach a level equal to that of the shake cultures at 96 hours. Interestingly, the YE static cultures were still growing at this time, unlike the shake cultures grown in the same medium.

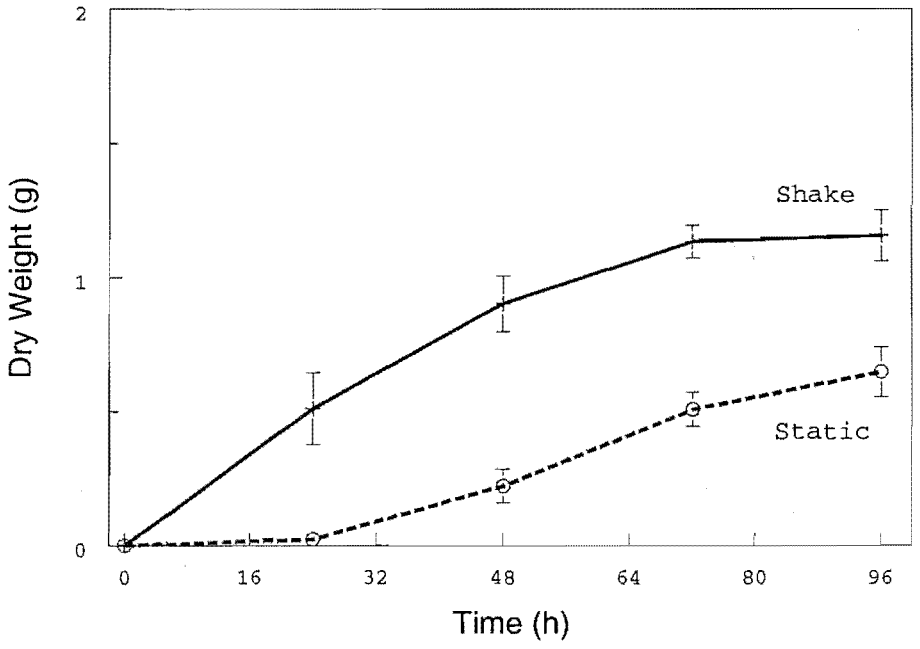


Figure 3. Dry weights of *Penicillium expansum* grown in ME medium.

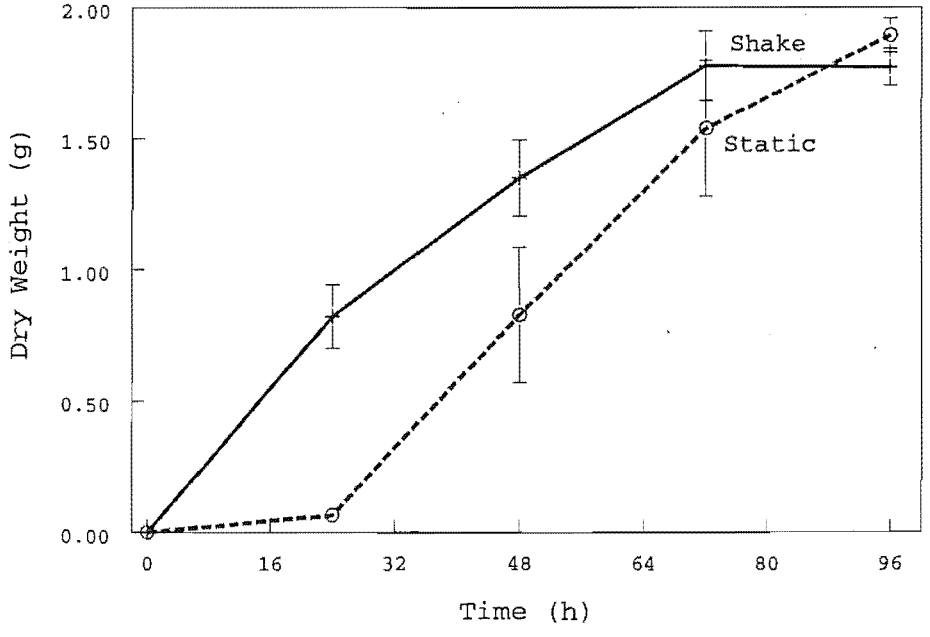


Figure 4. Dry weights of *Penicillium expansum* grown in YE medium.

3.3.2 Morphology.

The ME shake cultures started as a fine mycelial suspension at 24 hours, which progressively thickened after 48 hours and 72 hours. There was no apparent reduction in the visible viscosity of the culture through mycelial growth (Note that viscosity was not measured in these studies). By 96 hours small pellets, approximately 1 mm diameter, were visible. Brown pigmentation of the mycelium also occurred in these cultures.

The ME static culture showed almost no growth at 24 hours, and the growth that had occurred was present mainly at/on the sides of the flask at the surface of the medium. A fine layer had formed in patches on the surface in the 48 hour static cultures. This thickened (to less than 0.5 mm thickness) and spread over the surface of the medium, and had begun to sporulate at 72 hours. By 96 hours the mycelial mat was fully sporulating and covered much of the surface. This mycelia also showed some pigmentation although not as much as the shake culture.

The 24 hour old shake YE cultures grew as a mycelial suspension which thickened over the next 48 hours, causing a decrease in viscosity due to the large amount of mycelial material present. The mycelium also appeared to be growing in clumps, which were obvious when the culture was filtered. At 72 and 96 hours the suspension was sufficiently thick that the effect of agitation appeared to be significantly lowered. Some tiny pellets (smaller than 0.5 mm) were observed amongst the mycelial suspension at this time.

After 24 hours in the YE static cultures a mat had begun to form slowly, although there was mycelia submerged in the medium. By 48 hours the mycelial mat covered the surface of the flask, and parts of it appeared to be beginning to sporulate. The 72 hour culture mat had thickened further and was sporulating in most places. The 96 hour culture sporulated heavily and the mat thickness was approximately 1 mm.

Figures 5 and 6 show 3 day old shake and static cultures grown in ME and YE media respectively.

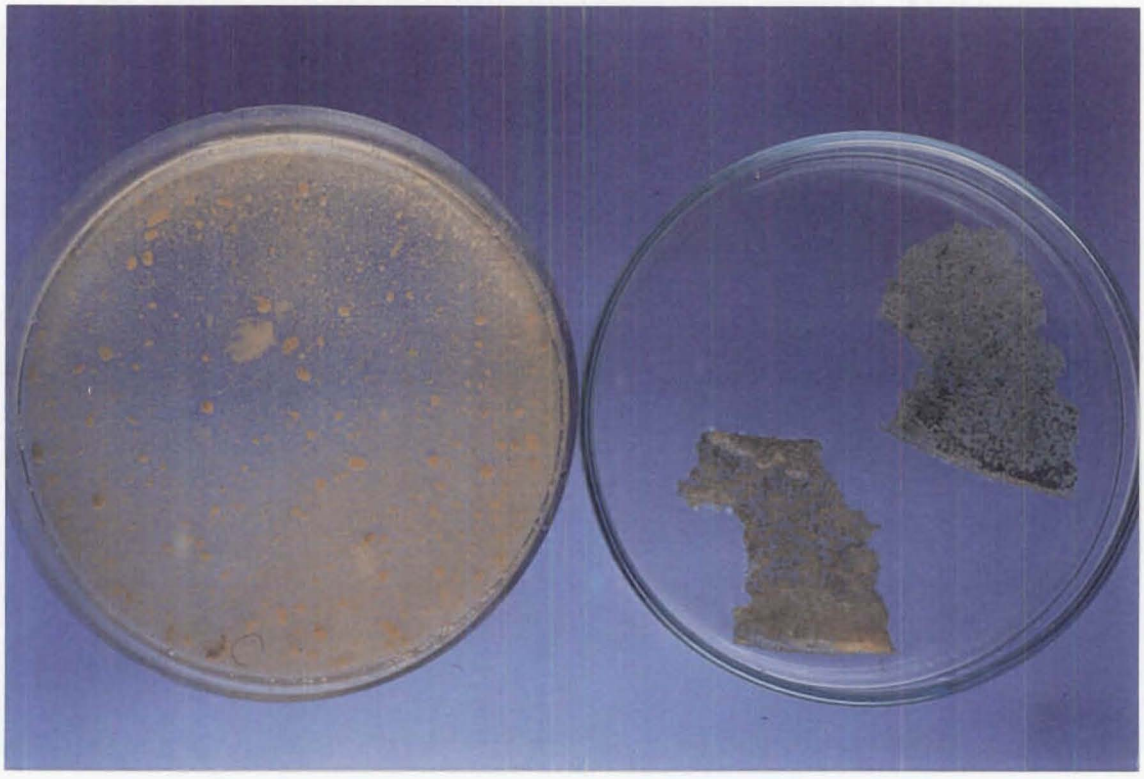


Figure 5. Comparison of shake (left) and static (right) 3 day old cultures grown in ME medium.

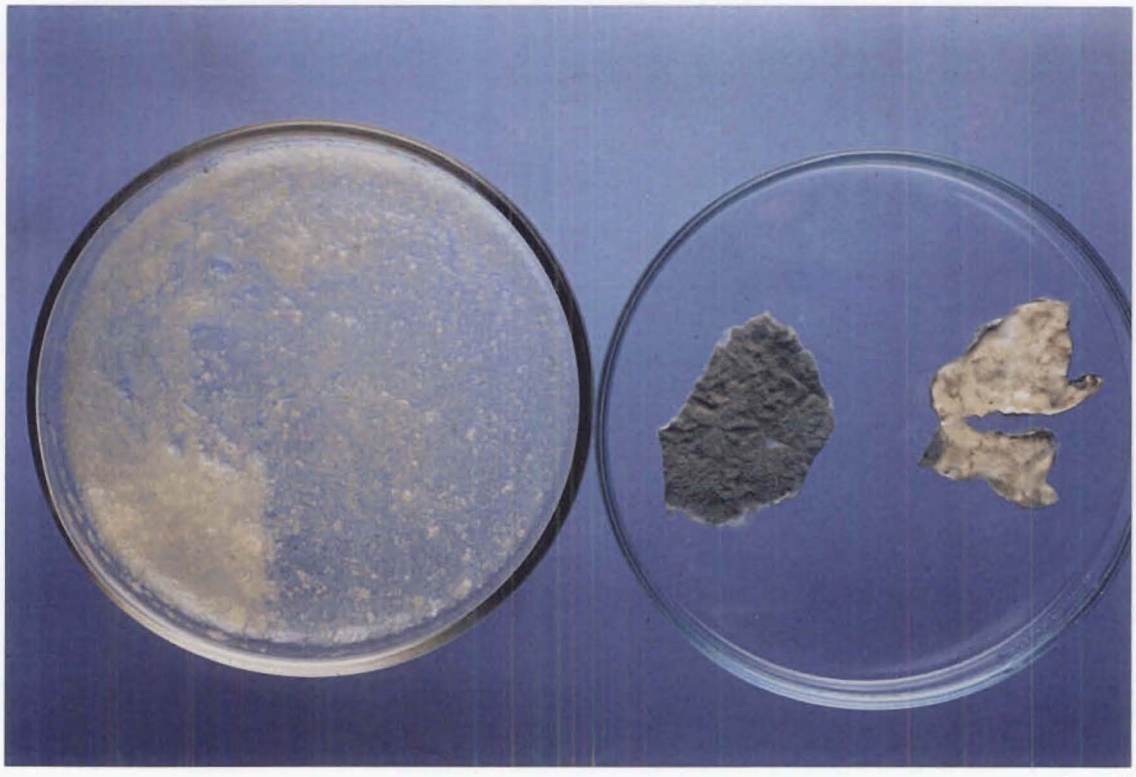


Figure 6. Comparison of shake (left) and static (right) 3 day old cultures grown in YE medium.

3.3.3 Conclusions.

Aeration, through agitation, had a significant effect on the growth of *P. expansum* in both the growth media used in this work. The effects were most obvious in the initial growth rates of the cultures where the static cultures showed almost no growth whilst the shake cultures underwent rapid growth. The composition of the medium affected whether the static cultures could catch up in growth rate. The glucose rich YEGB medium-grown static cultures displayed rapid growth once an initial critical size threshold was overcome and surface growth was well established, whereas the nutrient poor ME medium static culture grew at a slow rate and did not reach the same weight as the shake cultures.

The cessation of growth of the ME shake cultures could have been caused by a lack of nutrients, in particular the carbon source. As indicated in Chapter 4, a concentration of only 5 mM reducing sugars remained in the medium at 72 hours. The YEGB shake cultures still had an average reducing sugar concentration of 21 mM at this time, suggesting that another mechanism besides carbohydrate limitation might be affecting growth. Agitation of the cultures ensured that the mycelium remained completely submerged throughout, and the older YE shake cultures were very thick with mycelia as described. A possible explanation for the cessation of growth could be limitation of space (i.e. in the culture medium) in which the fungus was growing. The formation of aerial mycelia would not be possible since the organism was continuously submerged, compared to the static culture, which was able to extend above the surface of the medium, which was still growing at 96 hours. This may also explain the presence of small pellets in late YE shake cultures. The inability of the mycelia to grow further might result in concentrated areas of growth and aggregation, leading to pelleting. *P. expansum* could possibly fall into the category of "hyphal element agglomerating microorganisms" according to the definitions given by Nielsen (1996), reviewed in Chapter 1. This type of organism forms a clump of hyphal elements, through agglomeration, which eventually forms into a pellet.

Agitation of the cultures also had a very obvious effect on the morphology. In both types of medium, the static cultures formed differentiated mats on the surface of the

culture medium, which sporulated after a few days. The shake cultures maintained a more homogeneous growth form, although there were some pellets present in differing quanitities in both media (pellets were larger and more numerous in the ME cultures). The formation of pellets has been reviewed extensively in Chapter 1.1.2. The cause for pelleting in this case would possibly be due to nutrient status of the medium (Phillips, 1966). Pelleting was more prevalent in the ME cultures than the YE cultures and this could be the reason. Conidial concentration (Camici *et al.*, 1952) is discounted as a reason because both types of medium received the same size inoculum. Low agitation (Phillips, 1966) is another possible cause of pelleting. This may provide an explanation for the formation of small pellets in the older YEGB shake cultures. At this time the DO concentration would be significantly reduced because of the excessive mycelial growth, therefore providing conditions conducive to pellet formation.

Clumping occurred in the YE shake cultures i.e. mycelial aggregates which were apparent upon filtering the culture. This resulted in the reduced observed viscosity, which is possibly attributed to less well-defined and less rigid cell walls that occur due to oxygenation (Zetelaki and Vas, 1968). Fungal cultures tend to behave as non-Newtonian fluids (Banks, 1977), and this effect was observed here as these cultures grew thicker. The branched mycelial network forms a three-dimensional structure, which gives the suspension rigidity and results in a yield stress.

The static cultures formed surface mats and this provided the organism with an interface with O_2. It was necessary to include a wetting agent with the spore inoculum because the spores were very light and hydrophobic in nature. Because of this, once inoculated into the medium, the spores tended to float at the surface, promoting the formation of the surface mat. Whilst diffusion would appear to be less effective than agitation, the upper surface face of the culture is exposed. The other surface is not in contact with O_2 but is in contact with the nutrient medium at all times. The implications of this are discussed in the Concluding Discussion (Chapter 8) with respect to secondary metabolism in static cultures. This provides one significant difference to the shake cultures, where the same face of the mycelium contacts both the O_2 phase and the culture medium. Pelleting provides an interesting variation of

the surface culture since this gives rise to an internal environment, which can be depleted of both medium nutrients *and* O_2. This produces an environment conducive to the cessation of normal intermediary metabolism and the onset of secondary metabolism.

Brown pigmentation was observed in the malt extract cultures. This is possibly due to the activity of the oxidative browning enzymes *o*- and/or *p*-diphenol oxidase (DPO catecholase and laccase), commonly found in fungi and plants. These enzymes are responsible for the melanisation of tissues and utilise O_2 as a co-substrate. In some fungi, the production of DPOs is growth-associated, whereas others produce these enzymes during idiophase. The production of melanins occurs through the polymerisation of the reactive quinone products. The activity of extracellular laccase has been shown to coincide with the onset of fruiting body development in basidiomycetes (Wood, 1985).

It is possible that the production of DPOs in *P. expansum* occurred more readily in the ME medium because the lower nutrient levels encouraged the onset of secondary metabolism. The study of this aspect of secondary metabolism would have broadened the scope of this project significantly and therefore was not followed up. It is important to note the conditions under which this effect was occurring and to correlate it with polyketide biosynthesis.

CHAPTER 4

THE EFFECT OF AGITATION ON PRIMARY METABOLISM IN LIQUID CULTURES OF *PENICILLIUM EXPANSUM.*

4.1 INTRODUCTION.

In determining the effects of agitation and oxygen on secondary metabolism of *P. expansum* it is necessary to study their effect on primary metabolism which feeds and leads into secondary processes. This thesis was based on the earlier findings of Woodhead and Walker (1975), and the analyses performed in that work were expanded to include alternative pathways and possibilities in this study. The pathways and enzymes monitored are outlined in the following sections.

4.1.1 Respiration.

4.1.1.1 Glycolysis.

Glycolysis is a metabolic pathway found almost universally in biological systems. Glucose is converted to pyruvate, from which the energy molecule ATP is produced (via the tricarboxylic acid cycle and the electron transport chain). Glucose is derivatised with phosphate from ATP to form mono- and then diphosphate. The hexose carbon chain is then split into two 3 C units, glyceraldehyde and dihydroxyacetone phosphate, which are interconverted. Oxidation and then dephosphorylation of the glyceraldehyde-3-P occurs. The remaining phosphate is transferred to C2 from C3 and the 3-phosphoglycerate is dehydrated to form phosphoenol pyruvate. Pyruvate is then formed by removal of the phosphate and ketolisation. The two hexokinases, glucokinase and fructokinase, were studied to monitor glycolytic pathway activity. Glucokinase is a key regulatory locus in glycolysis since it catalyses the first step. This enzyme also feeds its product, glucose-

6-phosphate into the start of the pentose phosphate shunt. It therefore indicates the rate of glucose uptake. Fructokinase also indicates the feed of C6 compounds such as fructose derived from oligo- and polysaccharides. It was chosen in this study to monitor the proposed mannitol cycle (Hult *et al.*, 1980; Section 4.1.2.3), since it catalyses the phosphorylation of fructose generated by this cycle, for re-entry into glycolysis.

Phosphofructokinase is the key regulatory locus for glycolysis. It is subject to inhibition by fructose-1,6-bisphosphate, ATP, and citrate (present under aerobic conditions), and can be activated by fructose-6-P, ADP and AMP (present in anaerobic conditions) (Larner, 1971). It is key in determining whether the flux of carbon is glycolytic or gluconeogenic.

Pyruvate is converted to acetyl Coenzyme A by pyruvate decarboxylase. Acetyl CoA is the key intermediate in oxidative metabolism and in the synthesis of many cellular compounds. It provides the point at which fatty acids, isoprenoids and polyketides diverge from primary metabolism. Acetate is esterified to a coenzyme group (derived from pantothenic acid) which forms an activated form of acetate.

4.1.1.2 Tricarboxylic acid cycle.

The citric acid (or tricarboxylic acid (TCA) cycle, or Kreb's cycle) converts acetyl CoA to CO_2, coupled to energy generation by oxidative phosphorylation in the electron transport chain. The oxidation of one mole of pyruvate results in the formation of 15 ATP. Electrons generated by the TCA are carried by NADH and $FADH_2$ and transferred to via a series of electron carriers to O_2 and resulting in ATP generation. This pathway is the main cellular route for the utilisation of acetyl CoA. The TCA cycle takes place in the mitochondrion. Lipids and amino acids feed into this cycle through mechanisms such as β-oxidation and transamination. This pathway also provides important intermediates like α-ketoglutarate and oxaloacetate, which are in equilibrium with the amino acids glutamate and aspartate. Additional ATP is formed through the re-oxidation of $FADH_2$, produced with the oxidation of succinate. The marker enzyme for this pathway assayed in these studies was isocitrate dehydrogenase. The mitochondrial form of this enzyme utilises NAD as a coenzyme,

compared to the cytoplasmic form which uses NADP, which was also monitored during this study, with respect to an involvement in generating NADPH in *P. expansum*. The NAD-ICDH is positively regulated by AMP, reducing the K_m for isocitrate, as well as ADP. The enzyme is inhibited by ATP, adenosine diphosphate ribose and NADH by competing with NAD^+. An interlocking model for the two forms of ICDH is based on the NAD form being stimulated by AMP in order to promote the electron transfer of respiration, whereas the NADP form is more concerned with electron transfer in the synthesis of fatty acids (Larner, 1971).

4.1.2 Pathways for NADPH generation.

4.1.2.2 The pentose phosphate pathway.

The pentose phosphate pathway is a major metabolic route in many organisms and is responsible for the generation of NADPH for use as reducing power in biosyntheses and a number of cellular reactions. The pathway consists of two parts: the oxidative pentose phosphate pathway which is responsible for the generation of NADPH, and the non-oxidative part which produces pentose sugars and their derivatives for use in the production of other important biomolecules such as ATP, CoA, NAD^+, etc. Ribose-5-P and NADPH have been shown previously to determine the flow through the pentose phosphate pathway, by exerting control over the glucose-6-P dehydrogenase reaction (Muino-Blanco *et al.*, 1983). The first two reactions of the pathway use NADP as a cofactor, which differs from NAD because it contains an extra phosphate groups which allows it to be distinguished from the other by many enzyme systems. The pentose phosphate cycle cannot re-oxidise NADPH and therefore it must be consumed in reductive reactions outside the cycle.

Glucose-6-phosphate dehydrogenase was chosen to monitor the pentose phosphate shunt since it is the first enzyme of the pathway and key regulatory locus, catalysing the rate limiting reaction of this pathway. This enzyme has been isolated and characterised from a number of filamentous fungi. Glucose-6-phosphate dehydrogenases vary in size-the *Aspergillus parasiticus* enzyme was a 180 kDa protein composed of 4 equal subunits (Niehaus and Dilts, 1984), the thermophilic fungus *Penicillium duponti* enzyme had MW of 120 kDa (Malcolm and Shepherd, 1972). G6PD exhibits strict anomeric specificity, the enzymes from some sources act

at reduced reaction rates on analogues such as 2-deoxyglucose-6-P and galactose-6-P. The reverse reaction has proven difficult to evaluate since the product, δ-glucono-lactone-6-P is very unstable (its half-life is 1.5 minutes at pH 7.4) (Bonsignore and De Flora, 1972).

Isozyme studies of G6PD in various fungi have been limited with respect to changes in culture conditions and the studies by Woodhead and Walker (1975) are discussed with the results presented here. Wennekes *et al.* (1993) studied this enzyme from *A. niger* and *A. nidulans* and detected 2 bands on native and denaturing gels for both fungi. Muino Blanco *et al* (1983) used *A. oryzae* as the source and showed 2 isozymes were present when grown on glucose as the carbon source, with a third present when ribose was used as the carbon source, indicating it acts as a nutritional inducer for this additional isozyme. Positive interactions between sets of binding sites in *P. duponti* was believed to result in amplification of catalytic activity according to intracellular fluctuations of glucose-6-P and $NADP^+$, resulting in an potentially important regulation mechanism (Bonsignore and De Flora, 1972). Malcolm and Shepherd (1972) also showed that glucose-6-P protected G6PD from thermal denaturation.

6-phosphogluconate dehydrogenase was also assayed since it is the second enzyme of the pentose phosphate pathway and not subjected to the same degree of regulation as G6PD.

4.1.2.2 Malic enzyme.

Malic Enzyme is located in the cytoplasm and is involved in the transport of acetyl CoA between the cytoplasm and mitochondria. Since NADP is the cofactor for this enzyme it is a potential source of reducing power for the cell, particularly for fatty acid biosynthesis since it is closely associated with the TCA cycle and acetyl CoA metabolism. Although it is appears to be involved in the metabolism of pyruvate, malic enzyme does not have a clear cellular role. McCullough and Roberts (1974) showed that in *A. nidulans* malic enzyme is required for growth on carbon sources which are TCA cycle intermediates. From studies using acetate non-utilising mutants

it is also believed that this enzyme is induced by a C4 dicarboxylic acid to develop activity under conditions which induce in the wild type.

Wynn and Ratledge (1997) used an *A. nidulans* mutant lacking in malic enzyme to demonstrate that this enzyme is a major source of NADPH for the production of storage lipids in this fungus. The role of malic enzyme in cells is unclear, however it catalyses the decarboxylation of malate to pyruvate with the production of NADPH. Malate is the counter-anion for citrate efflux from mitochondria, thus affecting the supply of citrate to the cytoplasm at the same time as providing NADPH for fatty acid biosynthesis (Evans and Ratledge, 1985).

4.1.2.3 The Mannitol cycle.

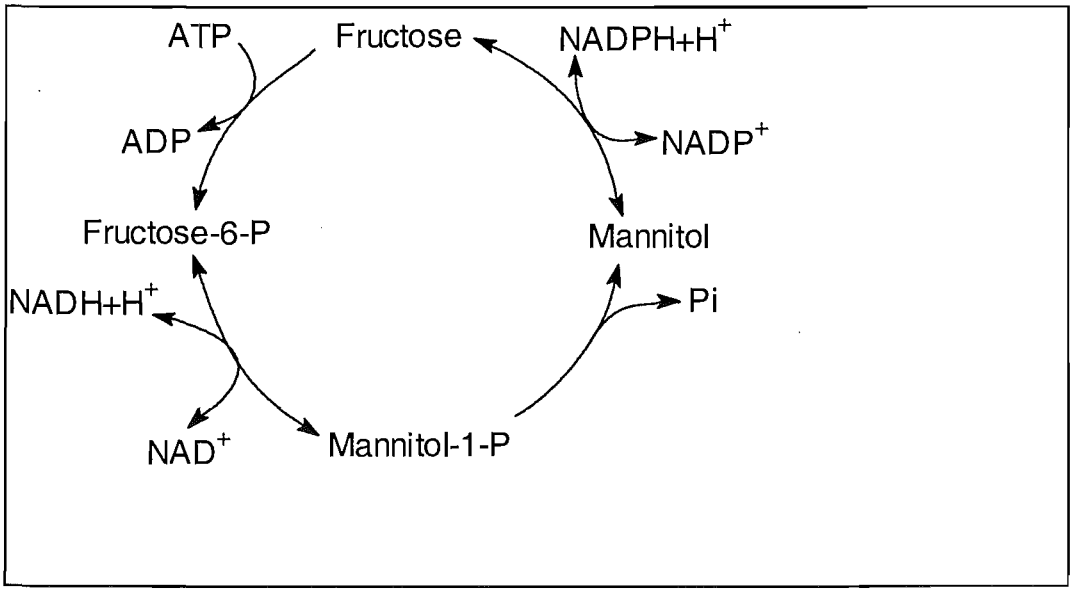


Figure 7. The mannitol cycle of *Fungi Imperfecti* (from Hult *et al.*, 1980)

Mannitol Dehydrogenase is a participant in the mannitol cycle proposed by Hult *et al.* (1980), and is the step at which NADPH is generated.

The Mannitol Cycle (shown in Figure 7) is proposed as an alternative means for the generation of NADPH in filamentous members of the *Fungi Imperfecti* (except for *Candida utilis*) and some of the Ascomycetes (Hult *et al.*, 1980). This cycle was shown first in *Alternaria alternata* and consists of the enzymes mannitol-1-phosphate

dehydrogenase, mannitol-1-phosphatase, mannitol dehydrogenase, and the fructokinase activity of hexokinase. The net equation for these activities is:

$$NADH + NADP^{+} + ATP \text{ ----------} > NAD^{+} + NADPH + ADP + P_i$$

Enough NADPH was produced in *A. alternata* to support its fat synthesis requirements. In a strain which produced lower levels of fat, but produced the polyketide alternariol, a lower level of mannitol oxidation was observed (evidence which suggested that polyketide production was favoured under limiting levels of NADPH) (Hult and Gatenbeck, 1978).

The availability of cofactors was shown to be the major factor regulating fluxes through this cycle. In a polyketide-producing strain, lower mannitol oxidation rates were shown to be unaffected by enzyme activities which were not limiting, and more related to the existence of an equilibrium between fructose-6-phosphate and glucose-6-phosphate mediated by mannitol-1-phosphate dehydrogenase and glucose-phosphate isomerase (of glycolysis) which were in *A. alternata* in high activities. This lead to the suggestion that the reactions of the Mannitol Cycle were involved in the maintenance of the redox states of the pools of NAD/H and NADP/H, and that they may have a regulatory function of carbohydrate metabolism (Hult and Gatenbeck, 1979).

Kiser and Niehaus (1981) showed that *Aspergillus niger* mannitol-1-phosphate dehydrogenase was highly specific for its reaction substrates and products - fructose-6-phosphate, mannitol-1-phosphate, NAD and NADH - as would be expected of the first enzyme of the cycle. This also confirms that early glycolytic reactions feed intermediates into the cycle as its only source, and that it operates in close association with glycolysis (as for the Pentose Phosphate Shunt). NADP-dependent mannitol dehydrogenase from *Aspergillus parasiticus* also showed limited substrate specificity. Its role in the cycle was shown to be important in experiments using Zn^{++} ions, which were a powerful competitive inhibitor of this enzyme with respect to mannitol (K_i = 1μM). Zinc inhibition resulted in the stimulation of versicolorin A (an aflatoxin

precursor) since Mannitol cycle activity was subdued, resulting in less NADPH production (Niehaus and Dilts, 1982).

None of the papers reviewed compared mannitol cycle enzyme activity with the pentose phosphate shunt, which is surprising considering the importance of the pentose phosphate pathway in terms of NADPH generation. Mannitol dehydrogenase reflects the metabolism of storage polyols (involved in carbohydrate storage since polyols are reduced forms of carbohydrates).

4.1.2.4 Cytoplasmic (NADP) isocitrate dehydrogenase.

Cytoplasmic (NADP) isocitrate dehydrogenase (NADP-ICDH) is an isoform of the TCA enzyme, not involved in the TCA due to its cytoplasmic location. Its main function is the generation of NADPH/ reducing power for fatty acid synthesis. Both forms of this enzyme are a primary site of regulation of respiration and fatty acid biosynthesis. The cytoplasmic enzyme catalyses the oxidation of isocitrate to 2-oxo-glutarate utilising NADP as a cofactor for the cytoplasmic form. Another key difference is that AMP is a positive allosteric effector of the NAD^+-utilising mitochondrial form. It should be noted that citrate levels regulate fatty acid biosynthesis without control of $NADP^+$-ICDH. (Larner, 1971)

4.1.3 Objectives.

The primary considerations in studying these enzymes and pathways can be summarised by the following questions:

1. What happens to primary metabolic flux under varying aeration and nutrient conditions?
2. Are specific pathways/enzymes activated in response to these conditions?
3. Are different isozymes produced under different culture conditions?
4. Do the electrophoretic properties of the protein alter? e.g. are the differences in the assayed enzyme activity due to regulatory alterations to the protein?
5. With respect to the inhibitor experiments, is there potential for specific compounds to act as allosteric effectors on glucose-6-phosphate dehydrogenase?

4.2 MATERIALS AND METHODS.

4.2. 1 Culture conditions

These are outlined in Chapter 3.

4.2.2 Reducing Sugar Assay.

The HBH (*p*-hydroxybenzoic acid hydrazide) assay developed by Lever (1972) was used. A suitably diluted aliquot (20 µL) of sample or glucose standard (10 mM in 0.3 % benzoic acid) was mixed with 5 mL HBH reagent (50 mM *p*-hydroxybenzoic acid hydrazide dissolved in 25 mM trisodium citrate containing 10 mM calcium chloride) and heated in boiling water for exactly 5 minutes. The assay mixture was cooled and the absorbance read at 430 nm.

4.2.3 Enzyme Assays.

4.2.3.1 Enzyme extraction.

Mycelium was separated from the liquid medium by filtration through miracloth, and rinsed with distilled water. The mycelium was ground to a fine powder using liquid N_2 in a mortar and pestle. This powder was added to chilled 0.1 M Tris HCl buffer pH 8.0 at a ratio of 1g: 4mL (quantities used depended on the number of assays performed with the extract, or the amount of mycelium available) and mixed to form a slurry, this resulted in a protein concentration in the final extract between 2.5-3.0 mg/mL. The slurry was centrifuged at 5000 rpm for 20 minutes at 4 °C. The supernatant was retained as the cell free extract used for each assay. To account for differing protein concentrations between extracts, the Bradford assay for protein estimation (Bradford, 1976) was performed on each extract and used to calculate specific activities (units per mg (U/mg) protein) for the enzymes monitored.

4.2.3.2 ELISA plate adaptations of enzyme assays.

The enzymes assays performed here were based on previous methods. In order to overcome the instabilities of the cell free extracts (assays had to be performed as soon as practical after extract preparation), a scaled-down version of these assays employing ELISA plates was developed. In every case, the primary enzymes selected for monitoring in this thesis could be assayed using a scaled down format based on that of Tsai *et al.* (1992) (with smaller volumes), and which utilised the change in absorbance at 340 nm due to reduction of NAD^+ and $NADP^+$. The same activity measurements were achieved as the 1.2 mL assays. The use of ELISA micro-titre plates was made possible by the plate reader used in this work (Labsystems Multiscan MCC/340, Douglas Scientific), which included a 340 nm absorbance filter.

The assays were performed by monitoring the change in absorbance per minute at 340 nm for the initial reaction rate. For the purposes of these studies, enzyme units were defined as $0.1U = \Delta A_{340}/min$. In each case the assay controls were by omission of the substrate, to account for any endogenous substrate in the cell free extract interfering or causing a reaction. 50 µL cell free extract was added to start, reactants were mixed by the plate reader for 5s then measured.

4.2.3.3 Glucose-6-phosphate dehydrogenase.

(Adapted from Tsai *et al.*, 1992) 125 µL 0.1M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$*, 62.5 µL 4 mM NADP, 62.5 µL 10 mM glucose-6-phophate (Na salt).

*The Mg^{2+} was omitted for assays investigating the potential inhibition of the ATP allosteric effect by this ion.

4.2.3.4 6-Phosphogluconate dehydrogenase

(Adapted from Tsai *et al.*, 1992) 125 µL 0.1M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$, 62.5 µL 4mM NADP, 62.5 µL 8mM 6-phosphogluconate (Na salt).

4.2.3.5 Isocitrate dehydrogenase (NADP)

(Adapted from Tsai *et al.*, 1992) 125 µL 0.1 M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$, 62.5 µL 4 mM NADP, 62.5 µL 8mM isocitrate (Na salt).

4.2.3.6 Isocitrate dehydrogenase (NAD)

125 µL 0.1M Tris HAc pH 7.6 containing 10 mM $MgCl_2$, 50 µL 10 mM isocitrate (Na salt), 50 µL 5 mM NAD, 25 µL 5 mM AMP.

125 µL 0.1M tris HAc pH 8.0 containing 20 mM $MgCl_2$, 62.5 µL 4 mM NAD, 62.5 µL 8 mM isocitrate (Na salt).

4.2.3.7 Hexokinase (glucokinase activity)

(Adapted from Tsai *et al.*, 1992) 112.5 µL 0.1M Tris HAc pH 7.4 containing 10 mM $MgCl_2$, 12.5 µL 40 units/mL glucose-6-phosphate dehydrogenase, 50 µL 10 mM ATP, 50 µL 5 mM NADP, 25 µL 0.1M D-glucose.

4.2.3.8 Hexokinase (fructokinase activity)

112.5 µL 0.1M Tris HAc pH 7.4 containing 10 mM $MgCl_2$, 12.5 µL 70 units/mL phosphoglucose isomerase, 50 µL 10 mM ATP, 50 µL 5 mM NADP, 25 µL 0.1M D-fructose.

4.2.3.9 Malic enzyme

125 µL 0.1M Tris HAc pH 7.4 containing 10 mM $MnCl_2$, 62.5 µL 10 mM L-malate (Na salt), 62.5 µL 4 mM NADP.

4.2.3.10 Mannitol dehydrogenase

125 µL 0.1M Tris HAc buffer pH 7.5, 62.5 µL 4 mM NADP, 62.5 µL 8 mM mannitol.

4.2.4 Bradford protein estimation

(Bradford, 1976) 0.1 mL sample was added to 5 mL Bradford dye reagent and mixed, absorbances were read after 2 minutes at 595 nm. Bovine serum albumin standards

ranging from 0.25-2.5 mg/mL were measured with each set of samples for calibration. The cell free extracts were diluted with 0.1M Tris HAc pH 8.0 buffer.

4.2.4.1 Bradford dye reagent.

100 mg Coomassie Brilliant Blue G dye dissolved in 25 mL 98 % ethanol, 42.5 mL concentrated phosphoric acid added, and made up to 500 mL with distilled water. This solution was shaken thoroughly and filtered just before use.

4.2.5 Isozyme analyses.

A vertical gel apparatus was set up and sealed with agar prior to pouring the separating gel. The gel was poured and allowed to set for 2 hours. A stacking gel was not used since the gels were run slowly overnight, allowing good resolution of bands when stained.

4.2.5.1 10 % w/v native separating gel.

16 mL H_2O, 12.5 mL 1M Tris HAc pH 8.8, 30 % acrylamide:bis solution (29.2g acrylamide, 0.8g bis in 100 mL dH_2O), were mixed together and degassed, then 300 µL 10 % ammonium persulfate and 15 µL TEMED were added to initiate polymerisation just prior to casting the gel.

4.2.5.2 Running conditions.

Gels were run in the cold room at 4 °C. The volume of sample loaded varied from 50 µL to 250 µL depending on the clarity/intensity of bands observed on staining (comparisons between samples were qualitative only, therefore no standardisation of protein concentration between the samples was done). The cell free extracts used for the enzyme assays were mixed with a 60 % sucrose solution (2:1 v/v extract:sucrose) prior to loading onto the gels. 10 µL Bromophenol blue (0.05 %) was used as the tracking dye for the anodal front. The running buffer was tris-glycine (4.54 g/L tris, 21.6 g/L glycine). The voltage was kept low when running overnight (usually <100V) so that good resolution of banding patterns could be achieved.

4.2.5.3 Isozyme staining.

Cell free extract samples from a 4-day culture run were loaded onto the same gel (samples were able to be frozen without affecting the banding patterns), to facilitate comparisons between samples.

The primary enzymes monitored in this work were stained with phenazine methosulfate and MTT coupled to the reduction of the pyridine nucleotides NAD and NADP, resulting in the formation of purple bands on a yellow background. This reaction was light sensitive and therefore it was necessary to stain gels in the dark (cupboard). Malic enzyme, isocitrate dehydrogenase and G6PD were the only three enzymes that could be detected. Two gels were run for each set of samples, one was stained with substrate present, the other omitted substrate in order to determine which bands (if any) were due to interfering dehydrogenases in the extracts.

4.2.5.4 Glucose-6-phosphate dehydrogenase.

40 mL 0.1M Tris HAc buffer pH 8.0 containing 10mM $MgCl_2$, 2 mL 8 mg/mL NADP, 2 mL 10 mg/mL glucose-6-phosphate (Na salt), 1 mg PMS, 4 mg MTT. Reagents were mixed together immediately prior to staining.

4.2.5.5 6-Phosphogluconate dehydrogenase.

40 mL 0.1 M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$, 2 mL 8 mg/mL NADP, 2 mL 20mg/mL 6-phosphogluconate (Na salt), 1 mg PMS, 4 mg MTT.

4.2.5.6 Isocitrate dehydrogenase (NAD^+ and $NADP^+$).

40 mL 0.1 M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$, 2 mL 8 mg/mL NAD or NADP, 2 mL 20 mg/mL isocitrate (Na salt), 1 mg PMS, 4 mg MTT.

4.2.5.7 Mannitol dehydrogenase.

40 mL 0.1 M Tris HAc buffer pH 7.5, 2 mL 8mg/mL NADP, 2 mL 20 mg/mL mannitol, 1 mg PMS, 4 mg MTT.

4.2.5.8 Malic enzyme.

40 mL 0.1 M Tris HAc buffer pH 7.4 containing 10 mM $MnCl_2$, 2 mL 8 mg/mL NADP, 2 mL 20 mg/mL L-malate (Na salt), 1 mg PMS, 4 mg MTT.

4.2.6 Glucose-6-phosphate dehydrogenase kinetic experiments.

The K_m and V_{max} of glucose-6-phosphate dehydrogenase from ME day 2 shake and static cultures were compared using the ELISA plate format outlined above (all volumes used remained the same). A series of glucose-6-phosphate concentrations (in the whole assay) ranging from 0.4-2.1 mM were assayed for change in absorbance at 340 nm per minute, and the specific activity (ΔA_{340}/min/mg protein) calculated by measuring the protein concentration using the Bradford method (Section 4.2.4). The results were analysed using the ENZPAK® program.

The same extracts were used to obtain the K_m and V_{max} of this enzyme using NADP as a substrate, except the glucose-6-phosphate concentration was held constant and a range of NADP concentrations (0.17-0.83 mM) were used. The results were analysed using the ENZPAK® program.

4.2.7 Glucose-6-phosphate dehydrogenase inhibitors.

A microtitre plate assay was devised based on that used in Section 4.2.3.2. 62.5 µL 0.1M Tris HAc buffer pH 8.0 containing 10 mM $MgCl_2$, 62.5 µL inhibitor, 62.5 µL 10 mM glucose-6-phosphate (Na salt), 62.5 µL 4 mM NADP. 50 µL cell free extract containing crude enzyme was added.

All inhibitors were used at 5 mM concentration: acetyl CoA, citrate (trisodium salt), fructose-1,6-bisphosphate, phosphoenol pyruvate, ribose-5-phosphate, 6-phosphogluconate, NH_4^+ (as ammonium chloride), glutamine, ATP, AMP, ADP, erythrose, NADPH.

The rate of reaction (ΔA_{340}/min) was determined for each inhibitor and compared to the original non-inhibited rate. Reactions that utilise NADP as a substrate which

might contribute to the overall reaction rate were performed excluding glucose-6-phosphate as the substrate to control for these enzyme activities in the cell free extract.

4.3 RESULTS & DISCUSSION.

4.3.1 Reducing Sugar Analyses.

The reducing sugar concentrations in both media were measured as an indication of the rate of sugar utilisation by *P. expansum* under shake and static conditions, and the potential for catabolite repression by glucose (or another carbohydrate) in explaining some of the activities and secondary metabolite production patterns. Discussion of reducing sugar levels in this Chapter are concerned with the effects on primary metabolism, particularly glucokinase, the first enzyme of glycolysis, and a key regulatory point for this pathway.

Figures 8 and 9 show the culture medium reducing sugar concentrations for ME medium and YE medium respectively. Results are expressed as glucose concentrations since this was used as the standard for the assay. The initial (uninoculated medium) reducing sugar concentration of ME medium was 6 fold lower than the YEGB medium initial concentration.

In the ME medium, the shake culture utilisation rate was approximately constant for the first 48h, slowing down significantly after this point as the concentrations became limiting to the culture. The static culture showed only a small decrease in carbohydrate concentration in the medium, the averages over the 4 days of culture growth changing by only 10 mM approximately. ME medium is made from 1.6 % w/v malt extract and 0.3 % w/v peptone. The carbohydrate composition of malt extract is given in Table 1. Peptone was included mainly as a nitrogen source, containing only 6.9 % carbohydrate, and contributing 0.207 g/L to the total ME medium carbohydrate concentration. Malt extract therefore contributed the carbohydrate content of ME medium. The carbohydrates derived from the malt extract base would arise from the malting of wheat and is likely to contain a number of different carbohydrates, predominantly mono-, di- and oligosaccharides, that are not necessarily detectable using a reducing sugar assay.

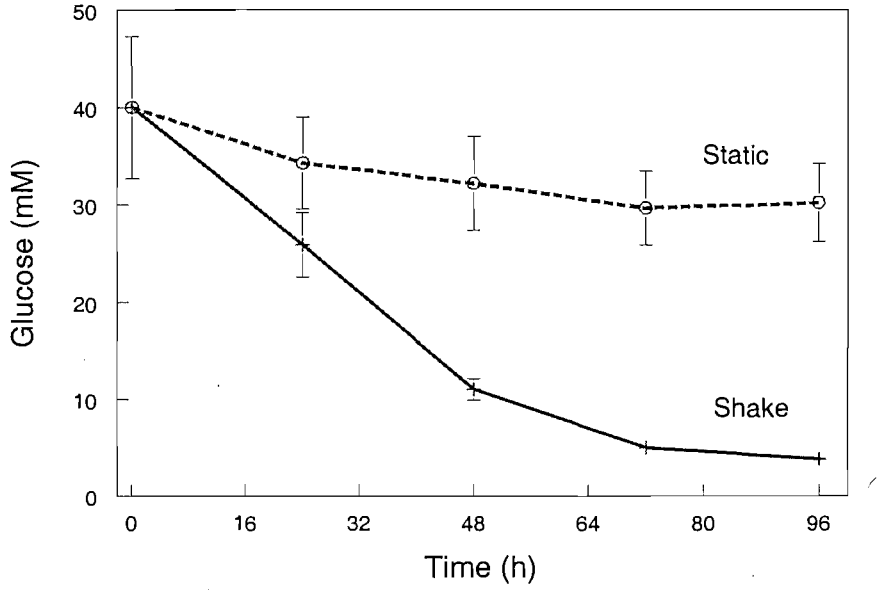


Figure 8. Reducing sugar concentrations in ME medium.

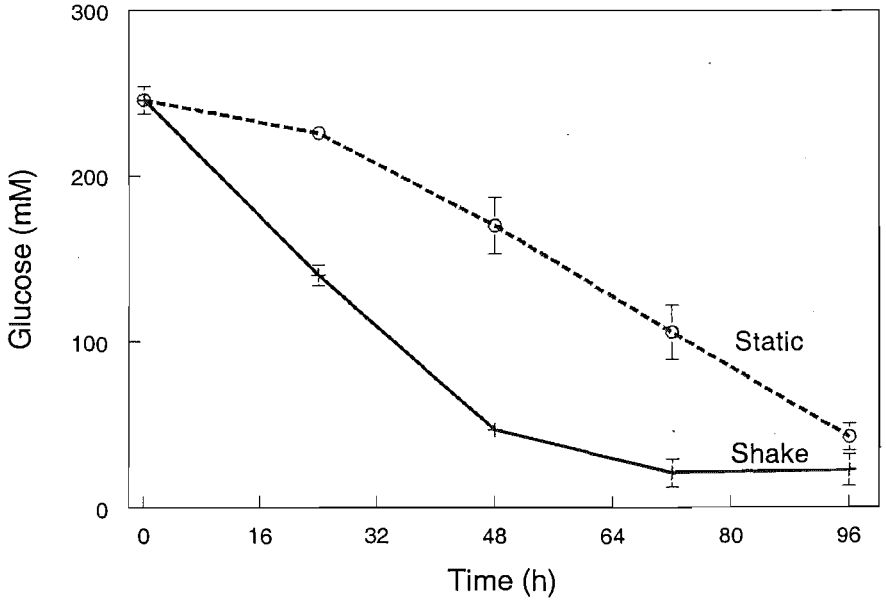


Figure 9. Reducing sugar concentrations in YE medium.

Table 1. The carbohydrate composition of Difco Malt Extract.

Component	Percentage in powder	Weight in ME medium (g/L)
Malt Extract base	40	6.4
Maltose	12	1.92
Dextrose (glucose)	40	6.4
Yeast Extract	8	1.28

Yeast extract medium, by comparison has glucose as the principal carbohydrate source, since it contains 4 % w/v glucose. Yeast extract makes up another 0.5 % w/v of this medium, of which 17.5 % is carbohydrate, contributing 0.875 g/L to the final medium carbohydrate concentration. It therefore follows that this medium has much greater reducing sugar concentrations than the ME medium. The rate of utilisation by the organism was also significantly higher. Once again the shake culture utilised the carbohydrate source more rapidly than static over the first 48 h, then slowing, with medium reducing sugar levels not changing at all between 72-96h. Since there would appear to be an adequate supply of reducing sugar remaining for continued utilisation (21 mM), it is possible there may have been a metabolic repression mechanism, or growth inhibiting mechanism occurring at this time, or alternatively, there could possibly have been some form of inhibition of the sugar uptake mechanism. This is discussed with respect to growth rates and other metabolism occurring in the organism in the Concluding Discussion (Chapter 8). The YEGB static cultures (after an initial lag period) utilised a great deal of the available carbohydrate also, albeit at a slightly reduced rate compared to the shake culture, but reaching a similar medium concentration by 96h.

The sugar transport systems of *S. cerevisiae* has been described (Garraway and Evans, 1984) and are considered representative of those in other fungi. These systems fall into two categories: inducible and constitutive. Constitutive systems involve carrier proteins always being present and an example of this is the non-specific pyranose system which takes up glucose (L and D), 2-deoxy-D-glucose, D-fructose, D-xylose, D-mannose, D-arabinose, D-lyxose and L-sorbose into cells. Inducible systems involve induction of protein synthesis and consequently a lag period during which

metabolism/synthesis is turned on, also known as adaptive growth, and require the absence of glucose. This includes the galactose system (induced by galactose) for the uptake of D-galactose, D-fucose, L-xylose, L-arabinose, and D-gulose; the disaccharide system which takes up D-glucose, maltose and trehalose; and the non-specific α-methyl-D-glucoside system which is responsible for the uptake of sugars such as D-glucose, maltose, trehalose, α-methyl-D-glucosides, and α-thioethyl-D-glucopyranoside. (Garraway and Evans, 1984)

There are also active and passive systems operating, in which active transport or facilitated diffusion can occur (respectively). Glucose can be taken up by both. The main factor affecting the uptake of sugar into cells is the presence of other sugars in the growth medium. Many sugars are mutually inhibitory since they may be competing for the same carrier molecule. Factors such as temperature, pH and inhibitors can affect proteins used for sugar transport. pH is of particular importance to the transport of sugars with protons. One example is maltose, for which the intake of 1 H^+ per maltose results in the release of one K^+ from the cell. Active transport mechanisms are also influenced by factors affecting respiratory activity, and the presence of inorganic ions such as K^+ which maintain the electrical neutrality during proton symport. (Garraway and Evans, 1984)

It is likely that a combination of some of these systems is operating in *P. expansum*. The rate of utilisation in the ME static cultures is likely to be affected strongly by slow metabolism causing a reduced production of energy molecules which aid in active transport mechanisms. Similarly, the lag periods in the static cultures could be explained by the presence of inducible systems, for which the induction mechanism may be overcome more rapidly in shake cultures. This is considered in the hexokinase activities (Section 4.3.2).

4.3.2 Cell disruption techniques.

A variety of cell disruption techniques were trialled to determine which yielded the greatest amount of protein in a cell free extract, and therefore by presumption, better enzyme activities. The Bradford assay (Section 4.2.4) was performed on each extract to compare the levels of protein released. The liquid nitrogen method (Section 4.3.2.1) adopted for extract preparation in the subsequent studies proved the best method. A 1:2 (w/v) ratio of mycelium to extraction buffer yielded extracts containing 2.25 mg/mL protein. The ultratarrex was also investigated as a means for cell disruption, using the same ratio of mycelium to extraction buffer, but only 0.2 mg/mL protein resulted in the extracts. Sonication of the mycelium in buffer gave negligible protein when assayed.

4.3.3 Hexokinase activities.

4.3.3.1 Glucokinase.

Figure 10 shows the glucokinase activities of shake and static cultures grown in ME medium. Shake and static activities were generally very similar, the standard errors for these cultures overlapping through the culture period. Aeration therefore appears to have no effect on glycolysis in this organism. Both cultures exhibited the same level of activity at the first 24h. The shake culture activity peaked at 48 h then steadily declined, whereas the static culture showed a constant activity level, declining gradually from 24 h. The similarities in activity show that there was a similar capacity for glucose uptake in shake and static cultures. Since there is a significant difference in the rate of glucose utilisation (Section 4.3.1), it can be concluded that although the initial sugar substrate concentration was low, the first step in its utilisation by glycolysis was not limiting if the activities of this enzyme are considered.

The same conclusion was reached by Woodhead and Walker (1975). They studied glycolytic activity in *P. expansum* by assaying aldolase activities in cultures grown in ME medium. Aldolase activities were the same in shake and static cultures, peaking at 48h, the decline after this time was attributed to the change to idiophase metabolism

from tropophase. The same isozymes were present throughout the culture period although these did differ in intensity.

Although there are overlapping standard errors between the YEGB shake and static cultures, activities in shake cultures tend to be higher than for static. The shake 48h peak average activity was higher than the static peak at 72h. Interestingly the 24h and 72h average activities were the same for shake and static. If one considers the difference in glucose utilisation rate at these times (faster for shake at 24h, and faster for static at 72h, see Figure 11), it can be concluded that another mechanism, instead of regulation of this first key regulatory step for entry of glucose into glycolysis, was operating to change the rate of sugar uptake.

It is therefore likely that the limitations to glucose utilisation in the ME static cultures and the initial YE static cultures were a consequence of another mechanism which is governed either directly or indirectly by agitation of these cultures. One proposed mechanism could be the transport of carbohydrate across the plasma membrane. Since ATP is produced by oxidative phosphorylation, the ability of the cell to generate it is linked to the availability of O_2. Therefore active transport mechanisms which utilise ATP will be linked to the presence of O_2, and it is possible that the lower oxygen concentration in the static culture could explain the slower initial transport/utilisation rate into the cells. It may also be possible that inducible systems may exist that are activated more readily in the presence of O_2.

4.3.3.2 Fructokinase.

This enzyme was included in these studies to investigate whether the proposed mannitol cycle was operating in this organism, as well as a comparison to glucokinase activity since it provides an alternative entry of metabolites into glycolysis. With respect to the proposed role in the mannitol cycle, the activites of this enzyme are considered with mannitol dehydrogenase in Section 4.3.3. Figures 12 and 13 show the fructokinase activities of *P. expansum* grown in ME and YEGB media respectively.

Fructokinase activity of cultures grown in ME medium were the same in shake and static over the first 24h. Beyond this time shake culture average activities were higher than static. Standard errors overlapped for each of the times monitored, however while the static cultures maintained a constant level of activity, the shake culture activities increased to peak at 72h, then decline to close to the static level. It is possible that the static level represents a base rate or that metabolites entering glycolysis at this point were fed at a constant rate and therefore enzyme activity was not required above this level. Since malt extract is derived from a plant source then it is likely that this enzyme does serve as an entry point for some sugars derived from this medium.

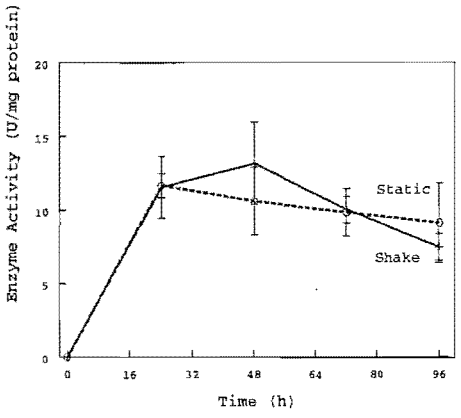


Figure 10. Glucokinase activity of *P. expansum* grown in ME medium.

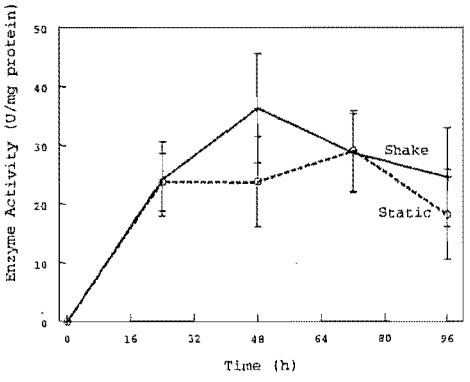


Figure 11. Glucokinase activity of *P. expansum* grown in YE medium.

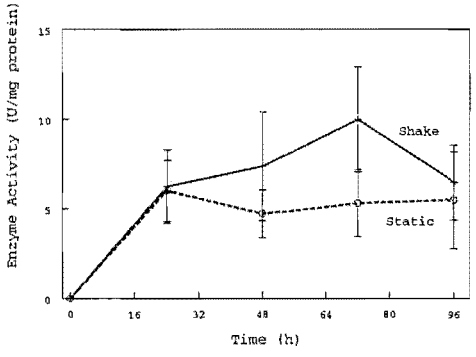


Figure 12. Fructokinase activity of *P. expansum* grown in ME medium.

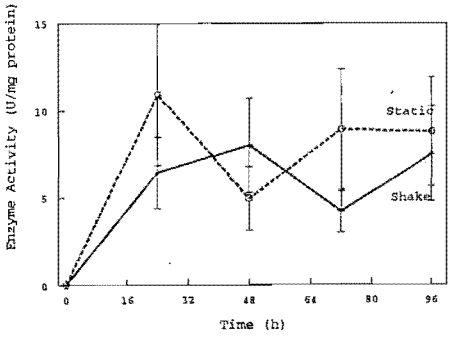


Figure 13. Fructokinase activity of *P. expansum* grown in YE medium.

Glucose forms the principal carbohydrate source in the YE medium, and the yeast extract would be expected to contribute only a small amount. Therefore, the supply of carbohydrates requiring entry into glycolysis through this enzyme is likely to be smaller. Considering this factor, the levels of activity of this enzyme in this medium might therefore be more representative of fluxes through the mannitol cycle than glycolysis.

Unilke the ME activities, fructokinase in YEGB medium exhibited some oscillations in both shake and static cultures. The activities were similar (standard errors

overlapped for each day), however static appeared to be generally greater than shake despite a significant dip in activity at 48h (where the shake activity was greater). These dips and oscillations are considered with similar patterns in mannitol dehydrogenase later in this chapter (Section 4.3.5).

The fructokinase activities were significantly lower than glucokinase activites in the respective media (glucokinase activities were doubled in ME and tripled in YEGB), indicating that glucokinase is the dominant means for carbohydrate feed into glycolysis (as would be expected). If the two enzymes are compared more closely, the static enzyme activities appear to show similar patterns in both ME andYE media i.e. constant activity from 24h. However the ME shake enzyme activities peak at different times (fructokinase later than glucokinase).

The two enzymes appear to have responded differently to the agitation conditions. ME glucokinase showed little difference between the average activities compared to fructokinase, which had slightly higher shake activities compared to static. Although reducing sugar levels were low in the ME cultures, the activities of this particular enzyme correlated well with the reducing sugar concentrations.

There is no definite correlation between the activities of fructokinase and glucokinase in YE medium. The shake cultures both appear to peak to 48h, however the activities do not compare beyond this. The 48h decrease in activity in ME is not seen in the YE cultures, however, at this time the YE activities had remained constant with the 24h results.

4.3.4 Pentose Phosphate Pathway.

4.3.4.1 Glucose-6-phosphate dehydrogenase activity in ME cultures.

Figure 14 shows that there was a clear difference in activity between shake and static cultures grown in ME medium. Over the first 24h the activities were almost the same, indicating that there was a similar flux capacity through this enzyme over this period between the agitated and non-agitated cultures. After 24h the static culture activity decreased slightly, but remained within a constant range. The shake culture activity peaked at 48h and then decreased to a similar level to the static cultures at 96h.

In terms of defining a period during the culture when a different capacity for NADPH production occurred, the 48-72h period would presumably result in greater NADPH generation in the shake culture compared to the static culture according to the activities observed here. Comparison of the activity curves for G6PD and glucokinase show that the activity of the former did not affect and was not affected by the activity of the latter. Shake and static glucokinase activities were more similar, however the activities of glucokinase and glucose-6-phosphate dehydrogenase peak at the same time in the shake cultures, and the shake cultures maintain relatively constant activity levels.

The isozymes of glucose-6-phosphate from *P. expansum* grown in ME medium were studied using native PAGE gels stained with PMS-MTT linked to the reduction of NADP by this enzyme when fed glucose-6-phosphate as a substrate. Figure 15 shows that there was a difference in banding patterns between shake and static cultures. The static cultures have 3 isozymes each, which were the same over the 4 days of the culture. Days 1 and 2 of the shake cultures had an extra band that ran faster than the static bands. By day 3 the faster of the bands had disappeared, and on day 4 the next fastest disappeared as well.

The possession of extra glucose-6-phosphate isozymes in the shake cultures, particularly on days 2 and 3 correlates with the higher enzyme activity observed in compared to the static cultures. The 96h shake activity was closer to the static culture activity on that day, and the loss of the 2 faster isozymes results in 2 remaining which run at the same place as the faster 2 of the static isozymes. The two fast isozymes of shake 24 and 48h cultures stained very rapidly compared to the others, suggesting that perhaps these have a greater affinity for the substrates compared to the other bands which ran more slowly.

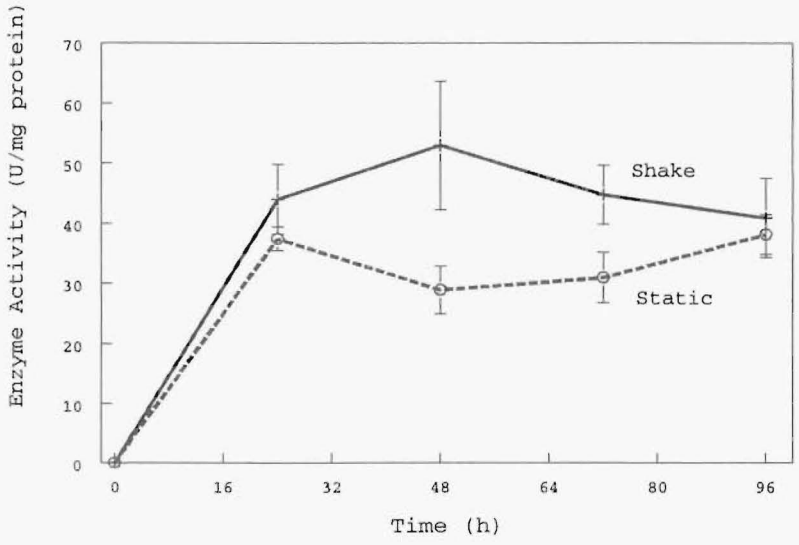


Figure 14. Glucose-6-phosphate dehydrogenase activity of *P. expansum* grown in ME medium.

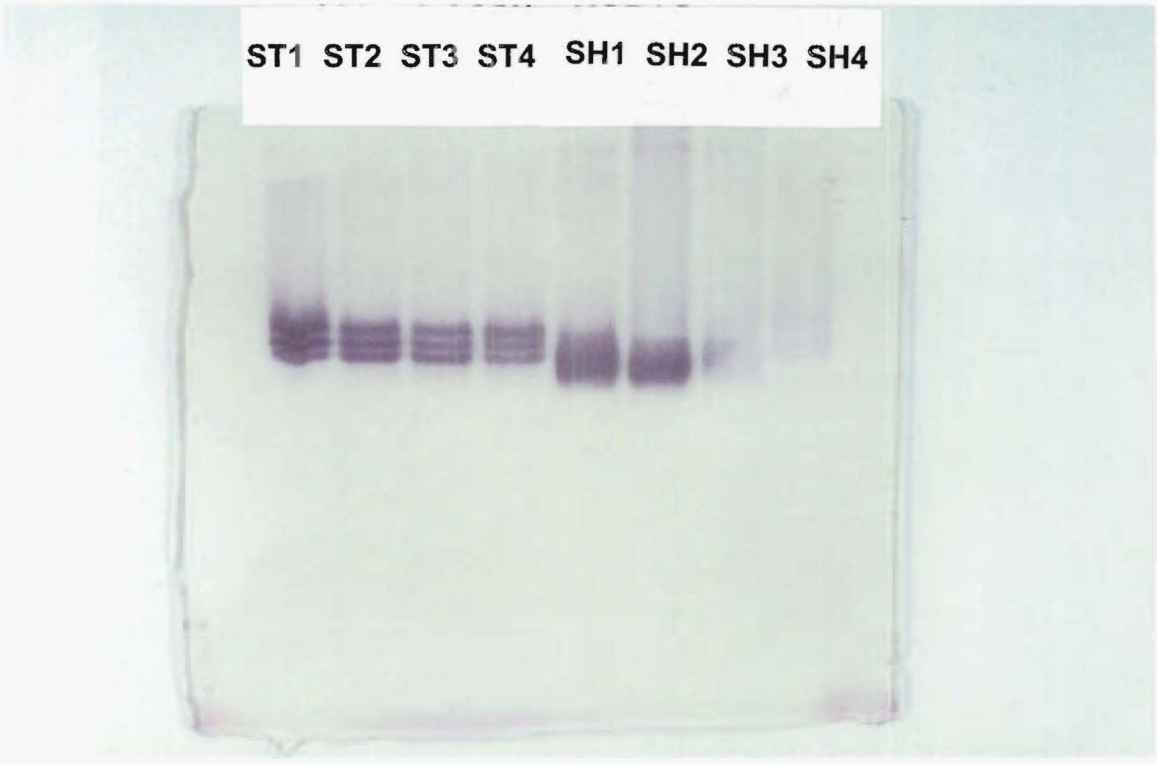


Figure 15. Native PAGE gel of glucose-6-phosphate dehydrogenase isozymes of *P. expansum* grown in ME medium.

The glucose-6-phosphate kinetics of the ME 48h shake and static cultures were compared since the activities and the isozyme banding patterns were significantly different. Crude extracts were used to compare Michaelis Menten (K_m) constants derived for the bulk crude activities for both substrates, glucose-6-phosphate and NADP. Tables 2 and 3 show the respective K_ms and V_{max}s for glucose-6-P and NADP calculated by several different methods using ENZPAK®.

In general, the shake culture K_m values were lower and V_{max} values higher than the static results for both substrates. This means than the 48h shake culture had a greater affinity for the 2 substrates since a lower substrate concentration is required to reach half of V_{max}. Similarly, the higher V_{max} shows that a faster rate of reaction is possible for the shake extract G6PD enzymes. This correlates well to the higher enzyme activity measured in the 48h shake culture, and the presence of 2 extra isoforms which appear to have greater affinities for the substrates given the speed of the staining reaction. Malcolm and Shepherd (1972) determined that the K_m values for glucose-6-P in *P. duponti* and *P. notatum* were 0.16 mM and 0.25 mM respectively, and that the NADP Km values were 43 μM and 62 μM respectively also. The glucose-6-P K_m values for *P. expansum* were in the same magnitude, whereas the NADP results were an order of 10 higher. A useful extension of this would have been to attempt to isolate the various isozymes in the 48h shake and static cultures in order to assess the relative affinities and kinetic characteristics as confirmation of some of these ideas. Inhibition studies might also provide some information about why particular isozymes are produced in response to specific culture conditions such as agitation.

Table 2. Glucose-6-phosphate dehydrogenase K_m and V_{max} values for shake and static 48h cultures using glucose-6-phosphate as the variable substrate as determined by various methods.

Method	Shake K_m (mM)	Static K_m (mM)	Shake V_{max}	Static V_{max}
Lineweaver-Burk	0.145	0.240	6.88	4.74
Hanes	0.268	0.437	7.50	5.38
Eadie-Hofstee	0.148	0.246	6.92	4.80
Direct Linear	0.543	0.872	8.62	6.53
Wilkinson	0.164	0.296	7.01	4.97

Table 3. Glucose-6-phosphate dehydrogenase K_m and V_{max} values for shake and static 48h cultures using NADP as the variable substrate as determined by various methods.

Method	Shake K_M (mM)	Static K_M (mM)	Shake V_{MAX}	Static V_{MAX}
Lineweaver-Burk	0.345	0.262	10.60	5.56
Hanes	0.172	0.320	8.20	5.99
Eadie-Hoftsee	0.130	0.270	7.70	5.64
Direct Linear	0.140	0.309	7.67	6.04
Wilkinson	0.178	0.314	8.46	5.94

The shape of the shake activity curve for this enzyme was very similar to that produced by Woodhead and Walker (1975), since the highest activities in those studies were also found in the 24 and 48 hour cultures. The static activities differed however, since the shake activities were reasonably low in the Woodhead and Walker experiments compared to the shake cultures, and this was not the case in the current study. The isozyme patterns were quite different for the two studies. There was a sequential decrease in the number of shake G6PD isozymes reported by Woodhead and Walker (1975) from 5 on day 1 to 2 by 96 h. There were 3 shake isozymes in this work, which did not change over the four days of the culture. The static isozymes were more similar to the Woodhead and Walker (1975) study since two isozymes were present over the 96 hours, although each day the second isozyme band increased in width, possibly through altered electrophoretic mobility. The isozymes in this study appeared to decrease or reduced in electrophoretic mobility. It should be noted that the types of electrophoresis used were quite different for each study. Woodhead and Walker (1975) used disc gel electrophoresis using 7.5 % w/v polyacrylamide, whereas this study used a 10 % w/v polyacrylamide vertical gel. This is likely to result in different electrophoretic mobilities due to the increased cross-links likely in the more concentrated gel.

4.3.4.2 G6PD inhibitor experiments in ME cultures.

A series of assays were run to find out what the effects of other cellular metabolites had on day 2 ME medium shake culture crude glucose-6-phosphate dehydrogenase activities. It should be noted that no comparisons were performed between the shake and static cultures, and therefore no conclusions can be drawn on the basis of

isozymes. Figure 16 gives a comparison of the G6PD activities in assays with different metabolites added. The strongest inhibition was achieved with NADPH which is consistent with previous findings (Levy, 1979), and indicates the role of this coenzyme in the regulation of this pathway. Malcolm and Shepherd (1972) showed that NADPH was a competitive inhibitor of both NADP and glucose-6-P in *P. duponti* and *P. notatum*. Although it could also be concluded that NADPH would affect the enzyme equilibrium, since these results appear to show the reaction has been completely reversed, Levy (1979) states that G6PD is thermodynamically irreversible. This would invalidate the latter scenario.

Strong inhibition was also achieved in these experiments with 6-phosphogluconate, the other reaction product. Once again, since G6PD is considered thermodynamically irreversible, this metabolite appears to serve as a strong inhibitor and therefore metabolic regulator, and not responsible for a change in the equilibrium. A possible mechanism could be deduced where accumulating levels of the reaction product in the cell (indicating slow or inadequate usage by the remainder of the pathway due to inhibition, etc), result in the inhibition of this enzyme. Ammonium and glutamine were also strong inhibitors of G6PD, which is interesting with respect to the repressive effect of these two compounds on secondary metabolism (discussed in Chapter 2), since inhibition here would tend to discourage NADPH production. Decreased NADPH levels would favour polyketide production over fatty acid synthesis. However, it should be considered that the repressive effect is likely to be mediated through another protein, and is unlikely to be operating in this medium because of the low ammonium concentrations; it would therefore operate separately. The effect seen in these experiments is more likely due to interaction by ammonium/amine/amino groups. It would have been interesting to see whether other sterically-correct, amine-containing molecules could achieve a similar inhibition to glutamine and NH_4^+. All three adenosine phosphates AMP, ADP and ATP, inhibited G6PD strongly. ATP inhibition of G6PD has been reported previously (Levy, 1979). Conclusions about the potential metabolic regulation of this pathway compared to glycolysis by ATP are not really valid because of the ADP and AMP effects, which would possibly indicate a lower energy state/less biosynthesis was occurring. Since

strong inhibition was achieved by all three, it could be concluded that there must be some structural feature of these molecules responsible.

Erythrose-4-phosphate was the other strong inhibitor of G6PD. This is consistent both with this compound being a pentose phosphate pathway metabolite therefore playing a regulatory role with accumulating levels, and with other work in which erythrose-4-P has been shown to inhibit this enzyme (Levy, 1979).

The other compounds investigated that gave significantly lower levels of inhibition included the glycolytic compounds fructose-1,6-bisphosphate, phosphoenol pyruvate and pyruvate. Accumulation of these compounds could not therefore be considered a mechanism to switch on the pentose phosphate pathway to compete for the glucose-6-phosphate common to both. Ribose-5-P, which is reported to be a regulator of pentose phosphate activity, gave only moderate inhibition as well. The effect of this metabolite on the pathway is not direct, and instead is mediated through the conversion of ribose to glucose (Peleato *et al.*, 1991). Acetyl CoA gave only moderate inhibition, indicating it is not a major effector in the regulation of this enzyme. Levy (1979) indicated that the reported regulatory effects of acetyl CoA were questionable due to the use of inappropriate assay conditions by the workers involved. Citrate (a TCA cycle metabolite) barely inhibited this enzyme. This molecule plays a significant role the regulation of glycolysis through inhibition of phosphofructokinase and pyruvate kinase, and its role in the activation of fatty acid biosynthesis.

In summary, the 6-phosphogluconate and NADPH are most likely to exert their effect in a competitive manner because of the structural similarities with the substrates glucose-6-P and NADP. The other strong inhibitors- glutamine, ammonium, ATP, ADP, AMP and erythrose-4-P- are more likely to operate as allosteric inhibitors, interacting with some other site on the enzyme rather than the active site, and less likely as non-competitive inhibitors which interact with the active site. This therefore implies that not only can regulation of activity occur through the presence of different isozymes as has been demonstrated, there is also the likelihood that metabolic

inhibition can occur as well since strong inhibition was demonstrated by several compounds.

The remainder of the potential inhibitors has only a small effect on the activity. Fructose-1,6-bisphosphate addition resulted in approximately 50 % of the uninhibited activity, with the others (acetyl CoA, ribose-5-P, phosphoenol pyruvate, pyruvate and citrate – respectively increasingly less effective) giving insignificant inhibition. No compound trialled acted as an activator, since no assay result gave a rate higher than that of the original.

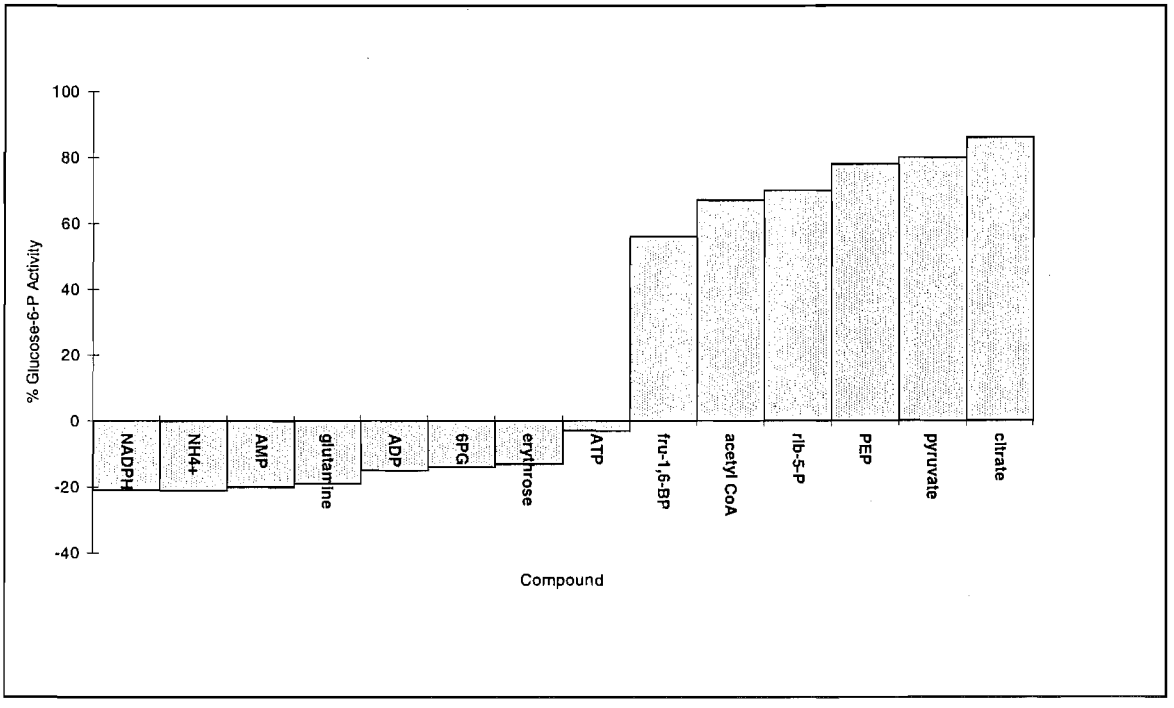


Figure 16. Effectors of the activity of *P. expansum* glucose-6-phosphate dehydrogenase grown in ME medium.

4.3.4.3 Glucose-6-phosphate dehydrogenase activity in YE cultures.

The YE shake G6PD activity curve (Figure 17) was very similar in shape to the ME shake curve, and activity levels were about the same for both media, indicating that the medium was not affecting the capacity for enzyme activity. This observation indicates that either the malt extract shake cultures were fully activated since they were at a similar level to the YE cultures, or that there may have been some catabolite

repression effect occurring due to the high sugar levels and rapid rate of uptake in the YE medium.

Activities in shake cultures were generally higher than in static cultures, however the standard errors overlapped, indicating that there were not significant differences in activities. This would possibly be due to similar control occurring over this enzyme despite the agitation difference between shake and static cultures.

The YE shake cultures followed the same pattern of activity to the glucokinase activities, i.e. peaking at 48h and then undergoing a gradual decline. The G6PD and glucokinase activities in the static cultures had different activity patterns, the glucokinase enzymes peaked at 72h, whereas the G6PD activities peaked at 24h and then underwent a gradual decline. It is possible that in static cultures the pentose phosphate pathway is preferred initially then switching to glycolysis as the preferred pathway for carbon metabolism. The activities of this enzyme in both ME and YE media were much higher than the hexokinase activities that this pathway would surely predominate. The flux of glucose-6-phosphate from glucokinase would not be at a high level given the enzyme capacity measured here and therefore the high G6PD levels seen here are likely to be regulated independently from the glucokinase activities, since there was also differences in the patterns of enzyme activity levels.

The glucose-6-phosphate dehydrogenase isozymes were studied using the same native PAGE system as the ME isozyme studies. The shake and static cultures both contained the same number of isozymes (3 each) which all ran to the same place for each day of the cultures. Two photos have been included (Figures 18 and 19) which show the same results with the exception of the 96h static culture in Figure 19. This culture appeared to possess an extra, faster isozyme, which may have been a modification of one of the existing isozymes through oxidation, or possibly in the handling of the extracts prior to electrophoresis.

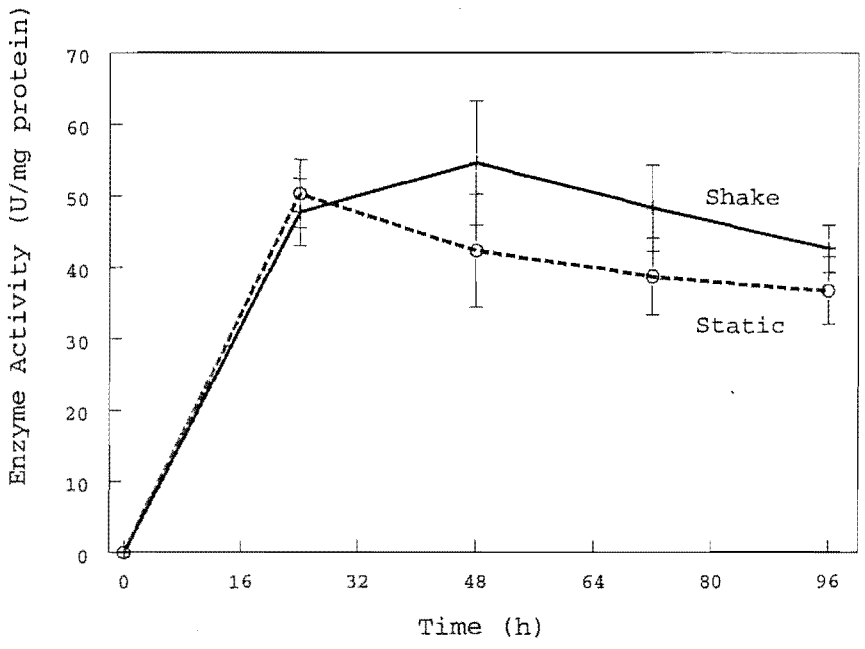


Figure 17. Glucose-6-phosphate dehydrogenase activity of *P. expansum* grown in YE medium.

Comparing the isozyme banding patterns with the corresponding enzyme activities gives an expected result. The shake and static cultures were quite similar regarding the levels of activities, and the isozymes found were the same. It can therefore be concluded that any differences in activity for the YE cultures were likely to be due to different amounts of the same isozymes rather than the possession of different isozymes. This is a significant difference between the ME and YE cultures with respect to the activity of this enzyme. PAGE to compare the electrophoretic mobilities of the ME and YE isozymes was not performed, but would have been useful in determining whether there were some common isozymes between the cultures grown in the different media. Generally, most cultures possessed (at least) 3 isozymes each, with the exception of the 96h shake culture. G6PD kinetic studies were not performed on any of the YE culture extracts since there were not significant differences in the enzyme activities or isozyme banding patterns.

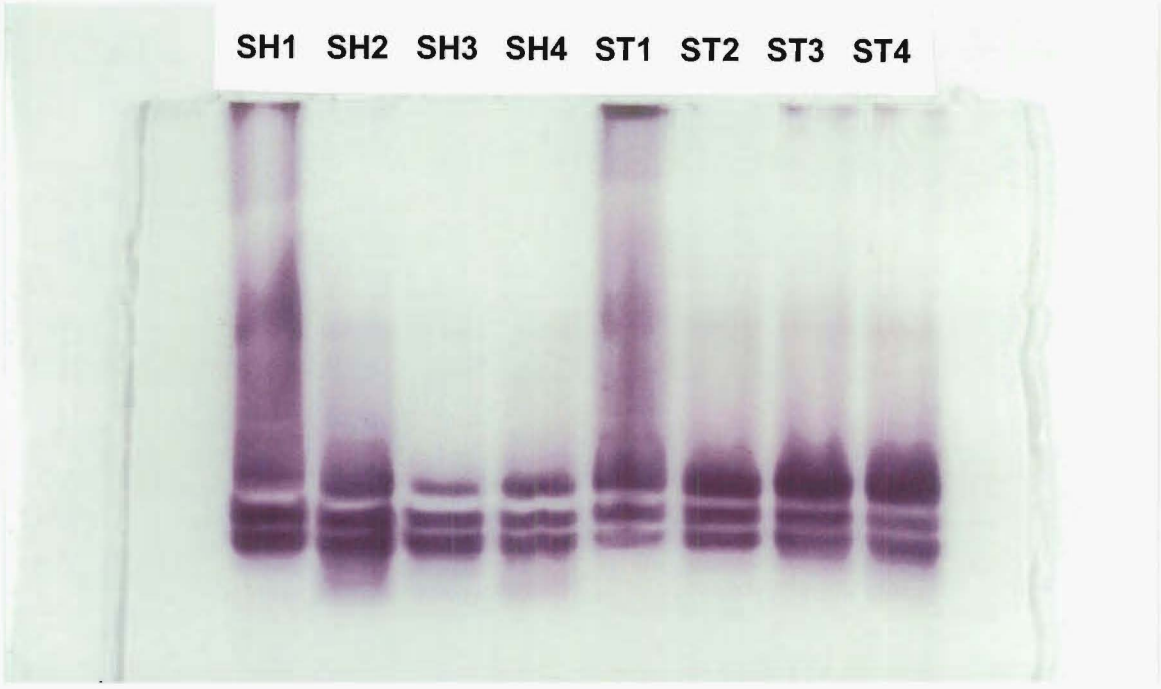


Figure 18. Native PAGE gel of glucose-6-phosphate dehydrogenase isozymes of *P. expansum* grown in YE medium – 1.

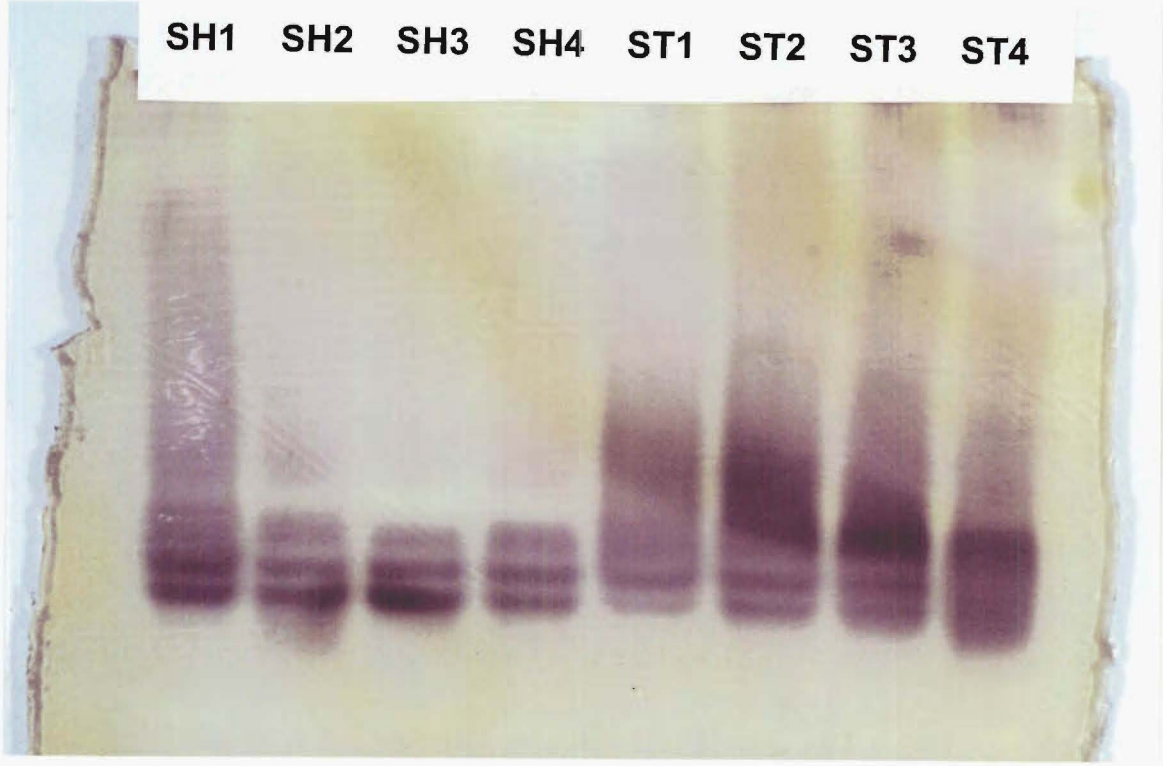


Figure 19. Native PAGE gel of glucose-6-phosphate dehydrogenase isozymes of *P. expansum* grown in YE medium – 2.

4.3.4.4 Glucose-6-phosphate dehydrogenase assays performed without Mg^{2+}.

A series of G6PD assays were performed using buffer that did not contain $MgCl_2$, in order to investigate whether the enzyme activity was significantly altered. Inhibition of G6PD by ATP has been shown to be reversed by the addition of Mg^{2+} since it was able to chelate ATP, therefore freeing the enzyme activity from this regulator (Levy, 1979). It would be expected therefore that the removal of Mg^{2+} ions would result in an increase in enzyme activity if there is an inhibition effect due to ATP. Morrison (1979) discusses the non-enzymic interaction between bivalent metal ions and nucleoside phosphates (particularly ATP). However, since this enzyme uses Mg^{2+} for its catalytic activity, it is possible that a reduction in activity could also be observed, unless endogenous levels are adequate to maintain the activity levels. It may also be possible that different isozymes were regulated to varying degrees by ATP, consequently affecting the Mg^{2+} interaction.

Figures 20 and 21 show a comparison of the G6PD activities with and without Mg^{2+} for cultures grown in ME and YE media respectively. In considering the results, it should be noted that the YEGB medium contained $MgCl_2$ added in a trace metals solution, giving a concentration of 0.05 % w/v in the medium, whereas there was no added Mg^{2+} in the malt extract medium.

The ME G6PD activities were very similar between the two sets of assays, indicating that the enzyme was unlikely to be inhibited by ATP in either of the shake or static cultures. The shake culture activities when Mg^{2+} was omitted from the assay were slightly higher than when it was included, whereas the opposite occurred (although to a very minor degree) in the static cultures. The overlapping errors tend to suggest that the differences were not significant except for the 96h static culture assay without added Mg^{2+}. This was lower than the assay containing Mg^{2+}, indicating that possibly there was not enough endogenous Mg^{2+} for the enzyme to catalyse the reaction to the same level as the assay in which it was present. This has interesting implications regarding possible inhibitory mechanisms for metabolism and growth, since it reflects the real *in vivo* situation as opposed to the assay conditions which ensure an excess of substrates and cofactors for the purposes of estimating enzyme levels and catalytic capacities. However the difference came about because the $+Mg^{2+}$ activity increased

at this time, rather than through a substantial decrease in the -Mg^{2+} assay of static day 4 activity.

The YE culture assays with and without Mg^{2+} were very similar, with standard error margins overlapping. The difference was slightly higher in the shake assays than in static ones, and the activity difference was greater between the shake cultures in the 72h cultures. The static culture assays were extremely similar, with the non-Mg^{2+} assay results being slightly lower.

The conclusion that can be drawn from this is that there did not appear to be an effect through ATP inhibition of G6PD. The only assay that showed a significant difference between added Mg^{2+} and omitted Mg^{2+} was the 96h ME static culture, and the possible reasons have already been outlined.

4.3.4.5 6-Phosphogluconate dehydrogenase activities.

6-Phosphogluconate is the second enzyme on the pentose phosphate pathway and therefore indicates the level of flux through the pathway from the perspective of an enzyme that is not the key regulatory locus.

The activities for this enzyme were low in ME cultures (Figure 22), with large errors around the averages. Shake activities were greater than static for the first 48 hours, with the peak at 24h. The activities were equal at 72h, then decreasing shake activity to 96h resulted in levels lower than the corresponding static culture. By comparison, the static activities remained at a relatively constant level throughout the culture period. It is possible that the static culture activities represent a constant level basic rate of an enzyme not under any specific regulatory influence, possibly a maintenance level.

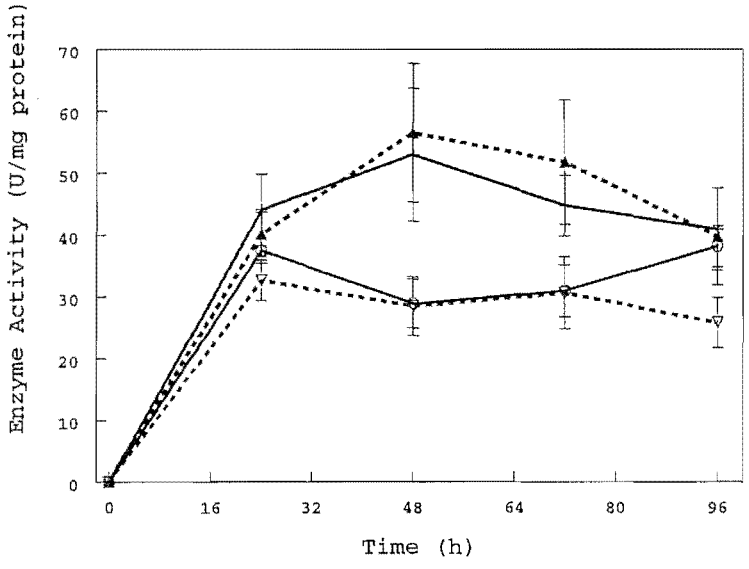


Figure 20. Glucose-6-phosphate dehydrogenase activities with/out Mg^{2+} added in *P. expansum* grown in ME medium. (+ = Mg added Shake, O = Mg added Static, ▲ = no Mg Shake, ∇ = no Mg Static).

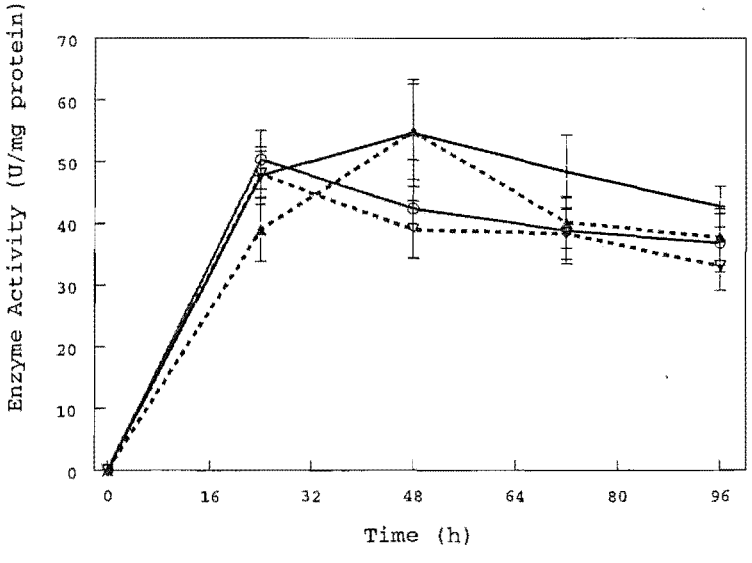


Figure 21. Glucose-6-phosphate dehydrogenase activities with/out Mg^{2+} added in *P. expansum* grown in ME medium.(+ = Mg added Shake, O = Mg added Static, ▲ = no Mg Shake, ∇ = no Mg Static).

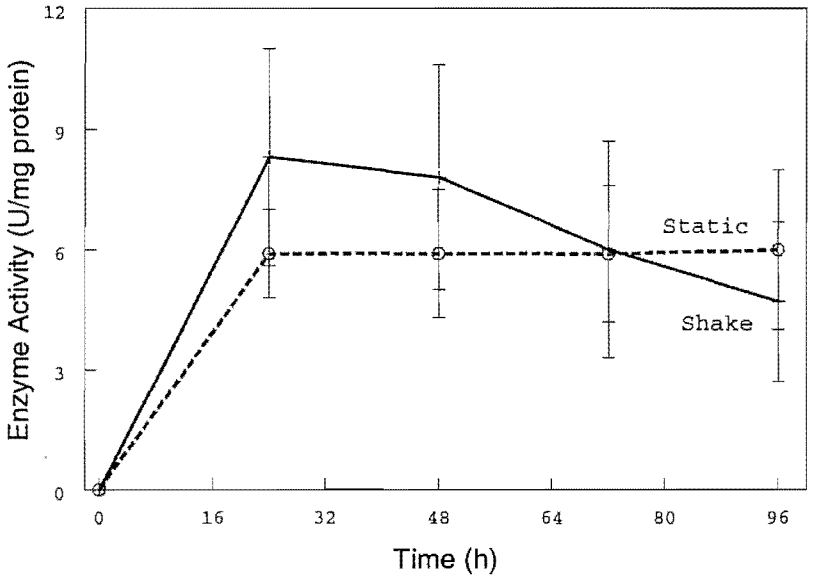


Figure 22. 6-phosphogluconate dehydrogenase activity of *P. expansum* grown in ME medium.

Comparison to G6PD activities reveal that the 6PGD levels were much lower. This is interesting considering the metabolism of glucose through this pathway would be governed by the ability of the lower activity enzymes to process the metabolites. The higher levels at the start of the pathway might reflect the reversibility of the reactions for both of these enzymes, but in particular the first committed step through G6PD. It is likely that G6PD maintained an equilibrium of a specific level of glucose-6-phosphate for the operation of this pathway, particularly in conditions that might favour glucose flux through glycolysis. This explanation is possibly more valid for cultures grown in this medium since the higher glucose concentrations of the YEGB medium would result in a greater flux of metabolites through both pathways.

The curves for static culture activities are similar since they remain at a relatively constant rate. The shake culture activity curves follow a similar shape although they are not identical: higher activities for the 24h and 48h cultures. The fact that the first

24h activities are slightly different for both enzymes indicates that flux through the 6PGD is related to the flux generated by the G6PD enzyme.

The YE culture 6PGD activities both increased towards the end of the 96h culture period (Figure 23). Shake activities were greater than static activities until 96h, when static activities were greater. Interestingly, neither culture increased between 24-48h (static activities dipped at this time). Although there appeared to be a significant difference in the levels at 48h and 96h, the standard error margins overlapped between shake and static cultures.

The shape of the activity graphs between G6PD and 6PGD activities for *P. expansum* cultures grown in this medium were quite different. G6PD activities were declining whereas 6PGD activities were increasing. It may be possible that the accumulation of the substrate generated by higher initial G6PD activities forced increased activity levels in 6PGD. Increased activation of 6PGD may have caused available metabolites to be pulled through the pathway as the culture period increased, to increase the activity capacity of the pathway. This may also be related to the equilibrium concept introduced in discussing the ME pentose phosphate pathway enzyme activities, with the balancing of metabolites being required later for 6PGD through the generation of glucose-6-phosphate substrate earlier through G6PD activity.

The 6PGD activity of *P. expansum* cultures studied by Woodhead and Walker (1975) was almost identical to the G6PD activity. This is not the case with this study as discussed already. However some similarities between the studies exist (referring to the ME culture results here). The 6PGD activity in shake cultures of both studies peaked at around 24-48 hours and then declined in activity, whereas the static culture activities increased throughout the culture period in both, peaking at 96 hours. The major difference between the 2 studies is that there is not much difference in activities in this study, whereas in the Woodhead and Walker study shake activities were distinct from static activities over the first 72 hours.

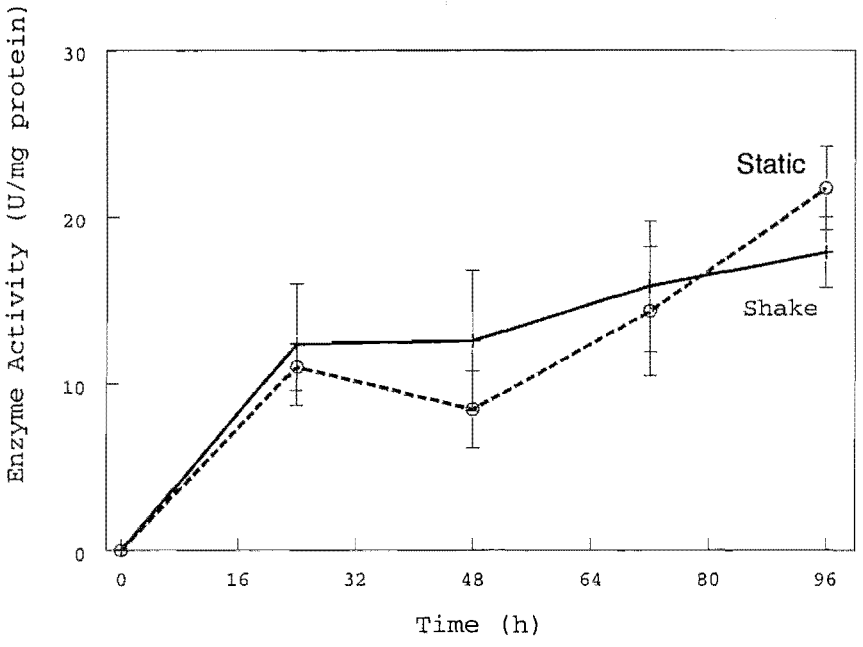


Figure 23. 6-phosphogluconate dehydrogenase activity of *P. expansum* grown in YE medium.

Direct comparisons between the two studies are difficult because the current study includes error margins and the assays performed were different in their composition. The Woodhead and Walker (1975) activities were considerably lower than those recorded here.

6PGD isozyme patterns were studied by Woodhead and Walker (1975) and it was interesting to note that there was a sequential decrease in isozyme activity in the shake cultures (as for the G6PD isozymes), indicating a loss of significance of this pathway over the culture period as it moved into tropophase. An extra isozyme appeared in the static cultures after 72 h as this pathway increased in significance under these conditions.

4.3.5 Mannitol dehydrogenase.

Figure 24 shows that the activity of mannitol dehydrogenase in malt extract medium was at low levels (around 4-5 U/mg protein) in both shake and static cultures. The activities in these two cultures were almost identical, with error margins overlapping. The shake activities increased slightly, then decreased at 96h, whereas the static activities were maintained at an approximately constant level. Given that the activity levels were so low, it can be concluded that either the proposed cycle was not operating, or that polyol metabolism was not significant in this particular medium.

When mannitol activity was compared to fructokinase, another enzyme involved in the proposed mannitol cycle, only static correlated in terms of the activity curve shape; both ME static activities were at a constant level. The shake fructokinase activities increased and the curve was a completely different shape to the mannitol dehydrogenase activities. When examining whether a relationship existed between glucokinase (glycolysis) and mannitol dehydrogenase (which branches off from glycolysis), a gradual decrease in glucokinase activity occurred over the culture period for both shake and static, whereas mannitol dehydrogenase tended to increase over the 4 days (except on day 4 in the static cultures). The expected observation would be for mannitol dehydrogenase to reflect glucokinase activity since the proposed cycle feeds off glycolysis at fructose-6-phosphate, the next enzyme in glycolysis after glucokinase.

The YE culture mannitol dehydrogenase activities were completely different to the ME cultures, as well as quite different between shake and static cultures (Figure 25). Although activities started out low in these cultures, they increased to levels double those of the ME cultures. Over the first 48h of the cultures, the shake activities were greater than static ones, however at 72h shake activities decreased (remaining at a reasonable level) and then increased back to the same level as at 48h. The static activities were at a constant low level for the first 48 hours, then appeared to be 'switched on' after this time increasing to 96h where the static activities were greater than shake.

When compared to fructokinase activities in the YE cultures, it can be seen that the oscillations in activity match i.e.

	24h	48h	72h	96h
Fructokinase	st > sh	sh ↑ > st ↓	st ↑ > sh ↓	st = > sh ↑
Mannitol dehydrogenase	st = sh	sh ↑ > st =	st ↑ > sh ↓	st ↑ > sh ↑

The arrows indicate whether activity increased, decreased or stayed the same (=), with the higher of the two cultures indicated. This shows that in general, the activities followed similar trends between the two enzymes. This may mean that there is a relationship between these two enzymes with respect to their participation in the mannitol cycle.

The activities of mannitol dehydrogenase in the shake and static cultures of both media appeared to show similar activity patterns to the activities of glucokinase over the first 72 h of the cultures. In that respect it would appear that there is a flow through effect from the glucokinase activity, and possibly this regulates the flux of metabolites into this mannitol cycle. The fact that there appears to be a greater level of correlation in the YE cultures compared to almost none in the ME cultures is probably a reflection of the increased concentration of metabolites produced under the more nutrient rich conditions.

In conclusion, the effect of agitation on mannitol dehydrogenase activity had the greatest effect in the YE medium, particularly over the first 48 hours of the culture. There was no effect seen in the ME medium and levels were generally very low. It would appear that the activity of the mannitol cycle, if it was operating, would make only a limited contribution to NADPH levels in the YE cultures (i.e. only in the first 48h), and given the level of activity, this would be secondary to the much greater levels of the pentose phosphate enzyme G6PD. No contribution would be likely in the ME cultures.

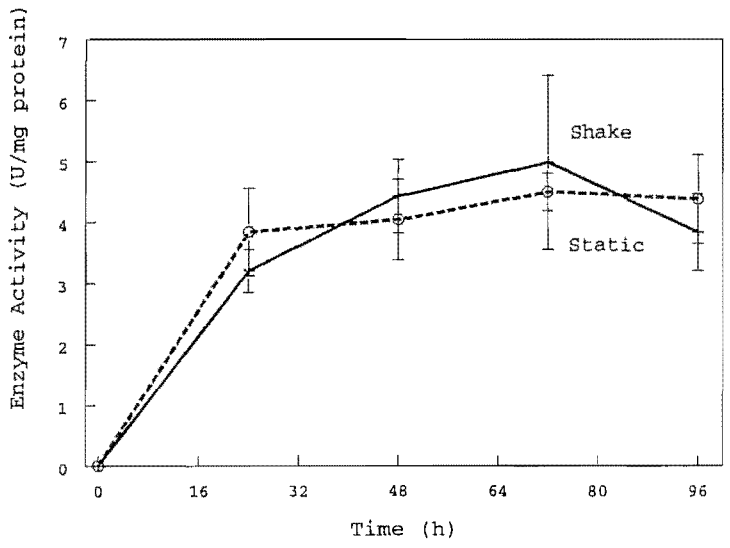


Figure 24. Mannitol dehydrogenase activity of *P. expansum* grown in ME medium.

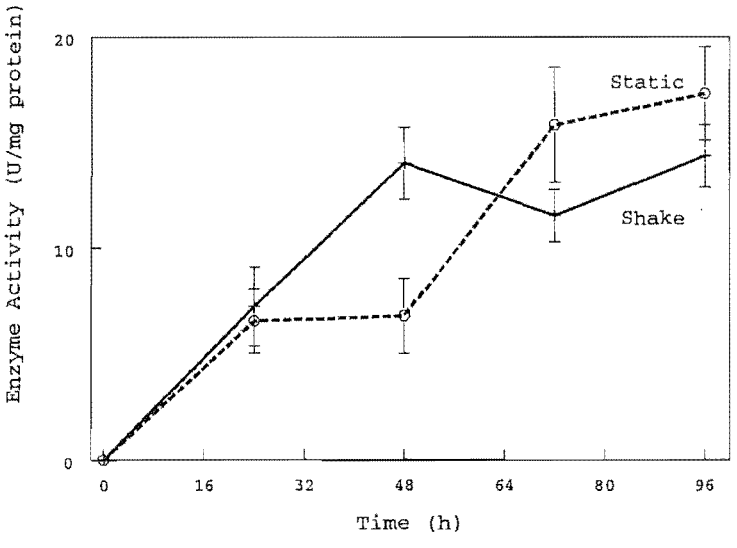


Figure 25. Mannitol dehydrogenase activity of *P. expansum* grown in YE medium.

4.3.6 Malic enzyme.

Malic enzyme is located in the cytosol, and is involved in a chain of reactions that transports acetyl CoA from the mitochondria to the cytosol, and is another proposed means for NADPH production.

The interesting aspect of the results of the assays for this enzyme was that the ME cultures had greater activity than the YE cultures. This is a significant reversal of most of the primary metabolism assays performed in this work. The activities in ME cultures (Figure 26) were still low, but shake was significantly greater than static over the first 48 hours (which was the peak activity), and then dropped off rapidly. Static culture activity increased gradually up to 72 h, where its activity equalled shake activity. Both cultures had almost negligible activity at 96h. Because the enzyme activities were so low, it is unlikely that the level of NADPH production is significant, although it may serve a localised effect. The malic enzyme activities in ME medium followed similar patterns to NAD-isocitrate dehydrogenase, except at 72h. The difference in the NAD-ICDH cultures is that the activities did not decrease to zero, and static activities continued to increase. Any correlation to TCA cycle enzyme activity would be expected because of the close relationship of malic enzyme substrate to TCA activity.

The YE medium malic enzyme activity was extremely low and only detectable at 24h where shake was greater than static (Figure 27). There was a significant degree of error in the shake culture averages, however this did not overlap with the static culture values. The low activities and the large standard errors prevent any meaningful analysis of these results. In view of the nutrient rich medium on which this culture was grown, other mechanisms rather than a lack of the enzyme presence should be considered. It would appear that some form of inhibition could be occurring in the crude extracts used to assay malic enzyme activity, especially since the native PAGE gels run showed the presence of enzyme at other times besides those assayed. The PAGE results are discussed later.

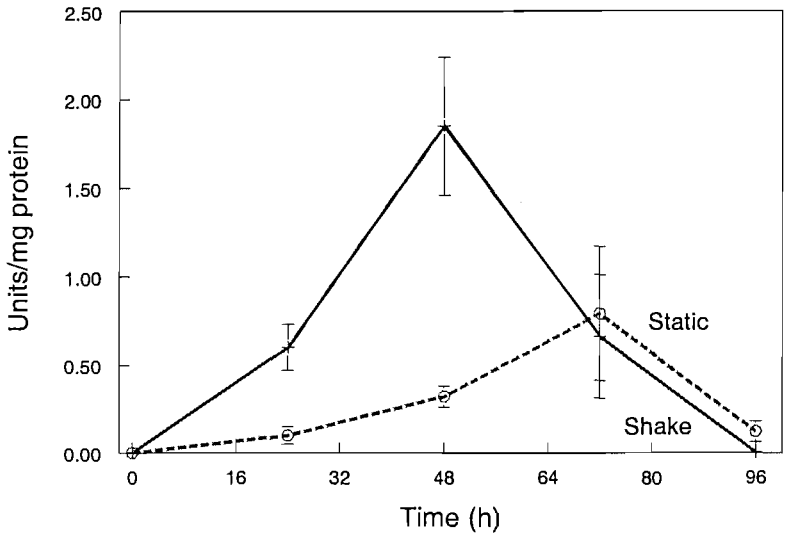


Figure 26. Malic enzyme activity in *P. expansum* grown in ME medium.

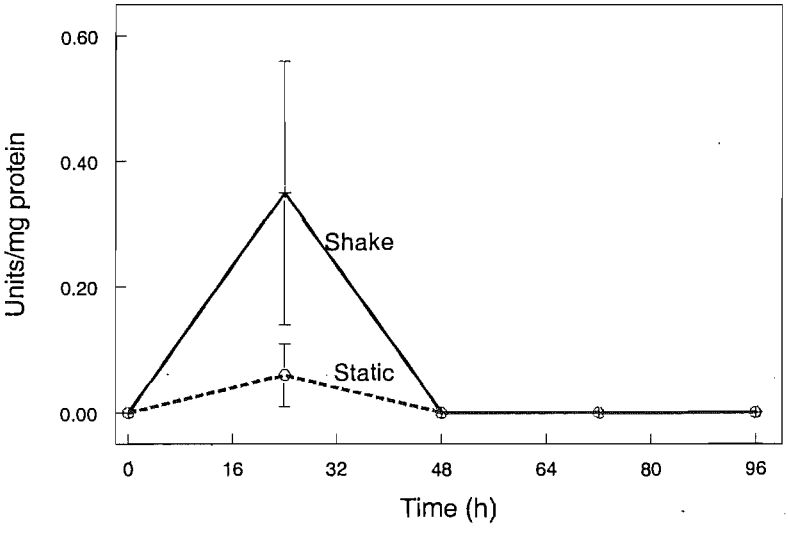


Figure 27. Malic enzyme activity in *P. expansum* grown in YE medium.

NADPH production by YE culture malic enzyme would be extremely insignificant in these cultures because the levels were so low. However the possibility exists that this enzyme has been inhibited or down regulated, and in particular this NADPH function could be part of the effect. Because activity could only be detected at 24h it is not

possible to draw relationships with the TCA enzyme assayed in these studies, NAD-isocitrate dehydrogenase. It is possible that the activity of this enzyme was inhibited according to the usage of the TCA cycle. Zink (1972) showed that the malic enzyme isozymes of *N. crassa* were repressed by pyruvate and acetate, and inhibited by aspartate, resulting in a decrease in the loss of C4 intermediates through decarboxylation. During early growth in sucrose medium malic enzyme would function to convert TCA intermediates to acetyl CoA. During later growth repression of the enzyme by acetate and pyruvate enzyme would occur.

Wynn and Ratledge (1997) also found only low levels of malic enzyme activity in *A. nidulans* despite claiming this enzyme as a significant NADPH source in this organism. They attributed this to the cultures being grown on glucose, and indicated that the activity would be likely to increase if transferred to acetate containing medium. As for *P. expansum*, the activities of the pentose phosphate enzymes were much greater than malic enzyme. These authors proposed that the role of malic enzyme would be significant if there was a separate cellular pool of NADPH produced by this enzyme which was preferentially channelled into fatty acid biosynthesis.

Native PAGE gels were run of crude extracts of *P. expansum* cultures grown on both YE (Figure 29) and ME media (Figure 28). Figure 29 is a representation of what was observed when the gel of the YE extracts was stained for malic enzyme. The gels were overloaded (250 μL) with extract in order to detect this activity. Interestingly, activity was detected in the YE gel for extracts in which no enzyme activity could be detected by the enzyme assay. It is possible that this staining system was more sensitive than the enzyme assays, however it may also be that electrophoresis separated out any inhibitors, therefore allowing the enzymes to be detected. However, given that the crude extract is diluted significantly in the enzyme assays, the inhibition by cellular compounds should be insignificant, in which case the sensitivity of the detection system would appear to be the reason for the bands showing on the gels. The isozyme patterns were the same for all the extracts.

Because there is no difference in the isozymes it is unlikely that the appearance or disappearance of isozymes contributed to the inhibition of the enzyme, and it is more

likely that some sort of allosteric modification was likely. It is possible that in view of the culture medium being rich in glucose that one of its catabolites may be involved in the mechanism. This is also possible since the same isozymes appeared in the ME cultures (note that the gel shown for the YE cultures in Figure 29 had overrun considerably compared to the ME culture gel shown in Figure 28). Evans and Ratledge (1985) showed that citrate, pyruvate, oxaloacetate and ATP only weakly inhibited malic enzyme, but no metabolite was found which exerted significant regulatory control over this enzyme.

The ME Native PAGE gel shown in Figure 28 was overloaded in order to facilitate detection, but this was at the expense of resolution of the isozyme bands. The gel therefore had common band(s) (possibly 2) at a R_f of approximately 0.54-0.59 (in relation to the anodal dye front). These bands were most visible in the shake day 1 cultures and all 4 days of the static cultures, and only slightly visible in the remainder (shake days 2-4). The intensity of the bands cannot be considered quantitatively since the protein concentrations of the extracts were not standardised prior to loading the gels (since detection was difficult enough as it was). It would appear that, considering the same isozymes are present between shake and static cultures in both media, the malic enzyme of *P. expansum* was very susceptible to modifications, likely to be allosteric in nature, which affect its activity but not its electrophoretic mobility.

The presence of the same isozymes in *P. expansum* throughout the culture differs from the malic enzyme isozymes in *N. crassa* (Zink, 1972). Isozymes 1 and 2 had a pH optimum of 8.0 and were present during the early stages of growth, disappearing after 24h, whereas isozyme 3 had a pH optimum of 10 and appeared at 12h, increasing throughout the culture period. These isozymes are also interesting in terms of the control of their formation by acetate and pyruvate. The pH 8 enzymes are repressed by acetate, whereas the pH 10 enzyme has greater activity in this substrate, and can be induced further by sucrose. Both enzymes were repressed by pyruvate.

Interestingly, the lack of substantial inhibitors of malic enzyme in the oleaginous yeast *R. toruloides* led Evans and Ratledge (1985) to conclude that the ultimate control over its activity would be substrate availability.

Figure 28. Native PAGE gel of malic enzyme of *P. expansum* grown in ME medium.

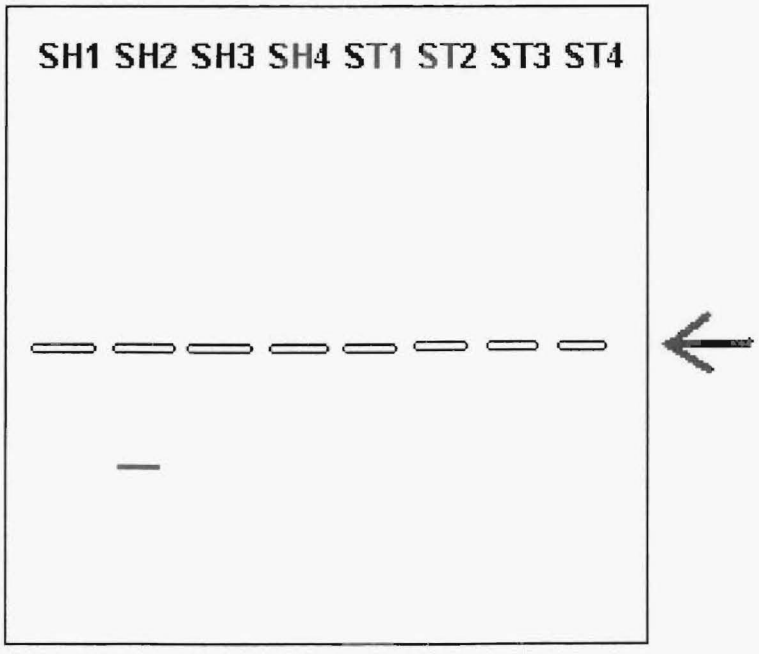


Figure 29. Native PAGE gel of malic enzyme of *P. expansum* grown in YE medium.

The complete lack of enzyme activity in *P. expansum* extracts despite the presence of the isozymes on native PAGE gels could possibly be explained in the same way. If the *P. expansum* enzyme is indeed allosterically regulated, it may be possible that the enzyme was not activated if there was no substrate available to signal a need for the activated enzyme.

4.3.7 Cytoplasmic isocitrate dehydrogenase (NADP form).

Figure 30 shows the results of the assays performed on *P. expansum* cultures grown in ME medium for cytoplasmic isocitrate dehydrogenase (NADP-ICDH) activity. The standard errors of the shake and static cultures overlapped, indicating that the activities were similar, however the shake culture average activities were generally higher than the static culture activities until 72 hours, when they were equal. After this time the shake culture activities decreased slightly whereas the static activities continued to increase. The activities in this medium were not high, although they were at a moderate level compared to other ME culture assays. This would indicate that there is a possibility that this enzyme could contribute to the NADPH pool of the cell, although, like the other enzymes implicated in this role, such as mannitol dehydrogenase, its activity would be secondary to that of the pentose phosphate pathway.

The activity levels do not appear to reflect those for the other cellular form of this enzyme, the NAD-utilising form which is present in the mitochondrion and participates in the TCA cycle. The shake culture activities did not resemble NAD-ICDH activities at all. Both forms increased activity in static cultures over the 96h period, although the NAD form was at much lower levels. It can be concluded that agitation did not have any real effect on the cytoplasmic ICDH activities in ME medium, and that the increases in the level of both shake and static cultures were more a reflection of the increased flux of isocitrate through the cycle, or acetyl CoA into the cycle later in the culture.

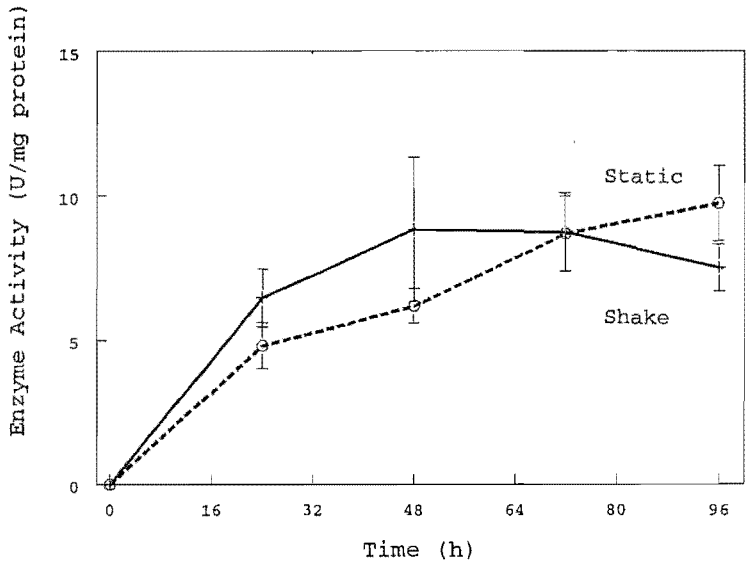


Figure 30. NADP-isocitrate dehydrogenase activity of *P. expansum* grown in ME medium.

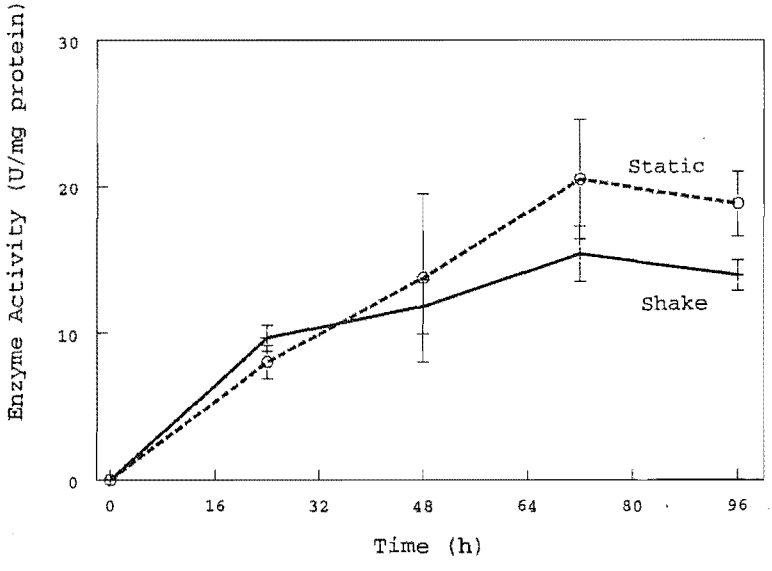


Figure 31. NADP-isocitrate dehydrogenase activity of *P. expansum* grown in YE medium.

Figure 32. Native PAGE gel of NADP-isocitrate dehydrogenase isozymes of *P. expansum* grown in ME medium.

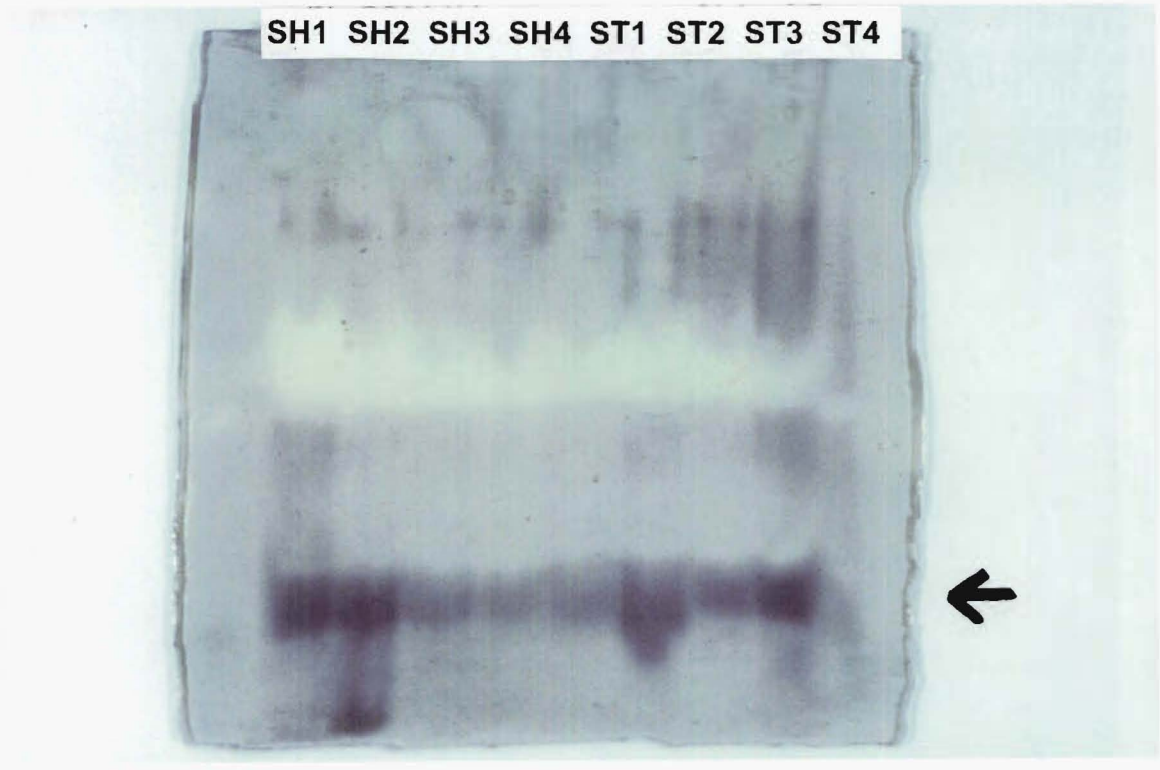


Figure 33. Native PAGE gel of NADP-isocitrate dehydrogenase isozymes of *P. expansum* grown in YE medium.

The activities of NADP-ICDH in shake and static cultures grown in YEGB medium were reasonably similar, particularly over the first 48 h of the cultures (Figure 31). At 72h the static culture activities were higher than shake (although the standard errors overlapped). By 96h the static culture activities were distinctly higher than the shake cultures, both types of culture having slight decreases in activity from the 72h peak. The level of activity in these cultures was reasonable, indicating that this enzyme may possibly have contributed to NADPH generation in this organism under these cultural conditions. It is not significantly different, except at 96h, to be considered a mechanism for switching on of secondary metabolism.

The interesting aspect of the activities of this enzyme in this medium and in ME medium is that towards the end of the culture period studied here, the static cultures had greater activity than the shake cultures. This was also the case in the mitochondrial isocitrate dehydrogenase activities, however, these activities were much lower. The YE NADP-ICDH activites did not resemble the NAD-ICDH activities, working completely opposite to this form. While increases were seen in the NADP form, the NAD form decreased. It is possible that this may have been due to competition for precursor, although in view of cellular location this may not be true. It may be possible that the respective sub-cellular pools were directed towards a preferred activity at a given time, i.e. the mitochondrial/TCA cycle form was more important at the beginning of the culture than at the end, and the NADP/cytoplasmic form became more important after 48h. However, conclusions such as this can only be tentative since the activity of the NAD form of this enzyme was about 5-fold lower than the NADP form.

A number of native PAGE gels were run to determine whether agitation or medium composition had any effect on NADP-isocitrate dehydrogenase isozymes. Most of these were unsuccessful and overloading of the wells was necessary in order to detect any form of activity. The gels that did indicate some activity are shown in Figures 32 and 33. It was difficult to determine which bands were responsible for activity in the ME culture extracts because those that were present were quite faint. A series of single bands were present at a R_f of approximately 0.43, however these coincide with banding present on control gels. A single band was present in the shake 48h extract,

which was also present in each of the YE culture extracts at an R_f of approximately 0.70. The resolution of the YE extract bands was poor, due to overloading in an attempt to determine what isozymes were present.

However, the presence of the same band in each YE culture extract was interesting. There was an extra band in the shake 48h culture with a much greater electrophoretic mobility than the others, and the static 48h culture appeared to also contain an extra, faster isozyme band, however this had a mobility closer to the common band. The presence of these two extra bands was unusual since the activity in both the shake and static 48h cultures was not dissimilar to activities on the other days. It may be that these two extra bands were a consequence of the handling of the extracts on that particular day, and can therefore be regarded as anomalies. There would not seem to be any reason for the organism to produce extra isozymes since the conditions at this time in the culture would not necessarily warrant a different isoform. The point in time where there is a difference in activity (96h) is not reflected in the isozyme banding patterns.

4.3.8 Mitochondrial isocitrate dehydrogenase (NAD form).

This enzyme was included in these studies because of its function in the Tricarboxylic (or Krebs) Cycle as an indicator of TCA activity in cultures of *P. expansum*. It also provided a comparison to another isozyme, the cytoplasmic NADP-requiring ICDH. Figure 34 shows that the activities of this enzyme in shake and static cultures grown in ME medium were at low levels. This enzyme showed a clear response to agitation in this medium, since shake cultures were distinctly different to static cultures. The static levels stayed low increasing gradually throughout the culture period. The shake culture showed significantly greater levels of activity, peaking at 48 h and decreasing to almost no activity at 96 h, slightly lower than the static culture activities at the same time.

The activity of this enzyme is influenced by agitation and therefore DO concentration because the TCA cycle is closely associated with the electron transport chain, which terminates with the reduction of O_2 to H_2O. The electron transport and TCA activity would have been important over the first 48 h in the shake culture, decreasing after

this, but relatively more important than in the static cultures. The static culture enzyme activities may have been limited by a lower precursor feed to this point in metabolism through a lower metabolic rate generally in these cultures. Chapter 8 includes a comparison with secondary metabolism.

The activities of NAD-ICDH in cultures grown in YE medium (Figure 35) were quite different to those of the ME cultures. The activities were almost equal for shake and static cultures throughout the culture period monitored. Both shake and static culture activities peaked at 24h, then decreased steadily for the remainder of the culture (although the shake 96h culture increased at this time). The standard errors for both these cultures were quite large over the four days indicating that there was variability between the runs. These results cannot be used as comparisons between shake and static, given the low results and the large standard errors.

The availability of terminal O_2 in the electron transport chain did not appear to be limiting in shake and static cultures compared to the ME static cultures. It is likely also that the nutrient rich medium ensured an adequate precursor feed to this point in metabolism. Alternatively, if there is an adequate precursor feed any effect due to O_2 availability may be overcome, or may not affect the TCA cycle activities if other metabolism is occurring at the same time, feeding from the TCA cycle (for example acetyl CoA feed to fatty acid biosynthesis, or transamination processes for biosynthesis in the cell.

Native PAGE gels for the detection of isozyme activities were unsuccessful. Some gels were allowed to stain for long periods in an attempt to find activity, however, the faint staining that appeared seemed to occur where the NADP-ICDH bands were detected, leading to the possibility that this was not the NAD-ICDH at all and that the NADP form had stained due to prolonged exposure to the substrate and staining solution.

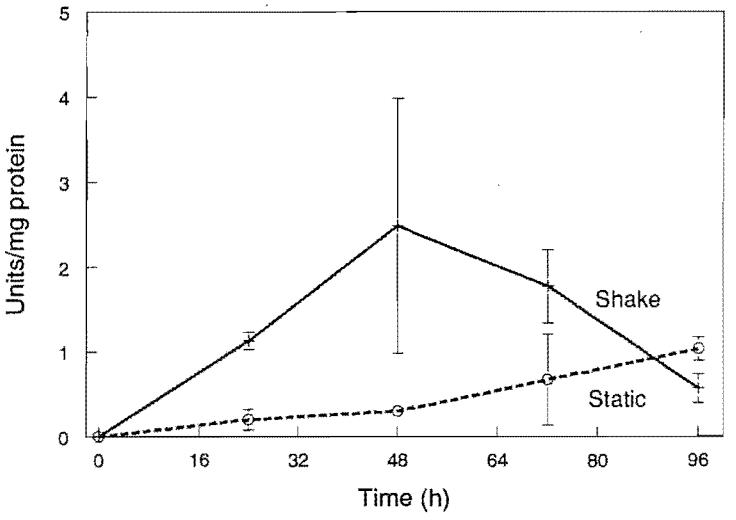


Figure 34. NAD-isocitrate dehydrogenase activity of *P. expansum* grown in ME medium.

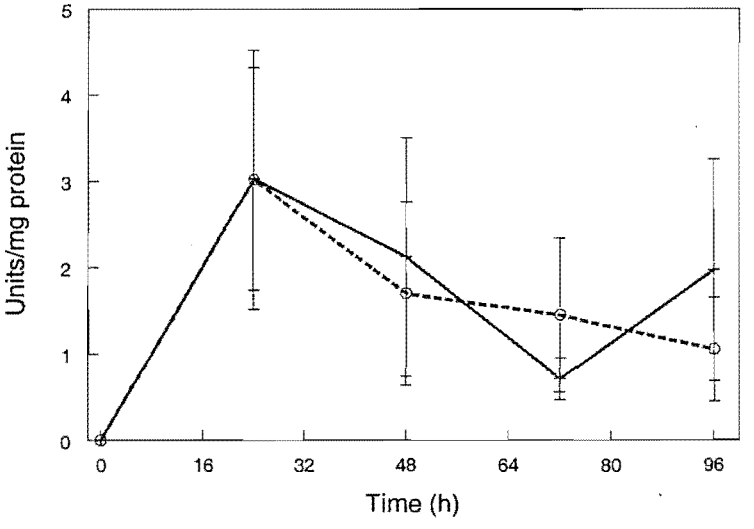


Figure 35. NAD-isocitrate dehydrogenase activity of *P. expansum* grown in YE medium.

Woodhead and Walker (1975) assayed succinate dehydrogenase to monitor TCA activity. In that study, the static culture activities were greater than shake activities through the culture period. The shake activities peaked at 48h, then decreased. The static activies peaked at 96 h, then decreased. By comparison, this study showed the ME culture TCA enzyme NAD-ICDH activities were greater in shake cultures than static although a similar trend of shake peak at 48h then decreasing, and static cultures increasing throughout the culture to peak at 96h. Once again, as mentioned in the 6PGD comparison, direct comparisons cannot be drawn since the assays were performed differently and in this case a different enzyme was used to estimate TCA cycle activity. The SDH isozyme patterns changed over the culture period for this organism in the Woodhead and Walker (1975) study, after 72 hours several isozymes were lost in the shake culture, whereas from 72 h in the static culture an extra isozyme was present.

4.3.9 Other isozyme activities.

The isozymes that were successfully stained have been discussed with the relevant enzyme assays (glucose-6-phosphate dehydrogenase, malic enzyme, NADP-Isocitrate dehydrogenase). However, as a consequence of the staining system used (phenazine methosulfate/MTT linked to NADP reduction), some staining effects separate to the enzymes studied were noted.

The first of these was the appearance of "nothing dehydrogenases" (Rothe, 1994), which give a positive staining reaction even when there is no substrate present. If allowed to stain for long periods, the gels studied here would often develop extra bands to those of interest. Determination of the "true" isozyme bands in this study was done by running two gels and staining one with the full stain recipe, the other omitting the substrate. These rogue bands were a particular nuisance for enzymes that were difficult to detect because of lower activities in the crude extracts that required overloading of wells in the gels. It is believed that alcohol dehydrogenases may be the culprits, since there are possibly traces of alcohol present as residues from the preparation of assay components. Note also that the extracts used in these experiments were crude cell-free preparations and not purified in anyway prior to running on the gels.

The second effect noted on isozyme gels stained with the PMS-MTT system was the appearance of clear regions on the gels against the purple background. The normal staining reaction involved the formation of blue-purple bands against a yellow background. However, as the gels were allowed to stain some background purple tended to form due to the exposure of the reagents to air. Clear yellow bands would frequently form on gels stained by including and excluding substrates and NADP/NAD. Rothe (1994) gives a staining procedure for the detection of superoxide dismutases (SODs) which utilises the PMS-MTT reaction. This allows the formation of a blue background through the reduction of MTT by reaction with PMS. The second step involves the reaction between reduced MTT and the superoxide radical (O_2^-) to produce MTT which is colourless, creating the yellow bands on the gels. This effect can be seen in Figure 36, in which shake and static YEGB culture extracts were run on native PAGE gels and stained with PMS and MTT and pH 8.0 50 mM Tris buffer, without the inclusion of other enzyme substrates or cofactors.

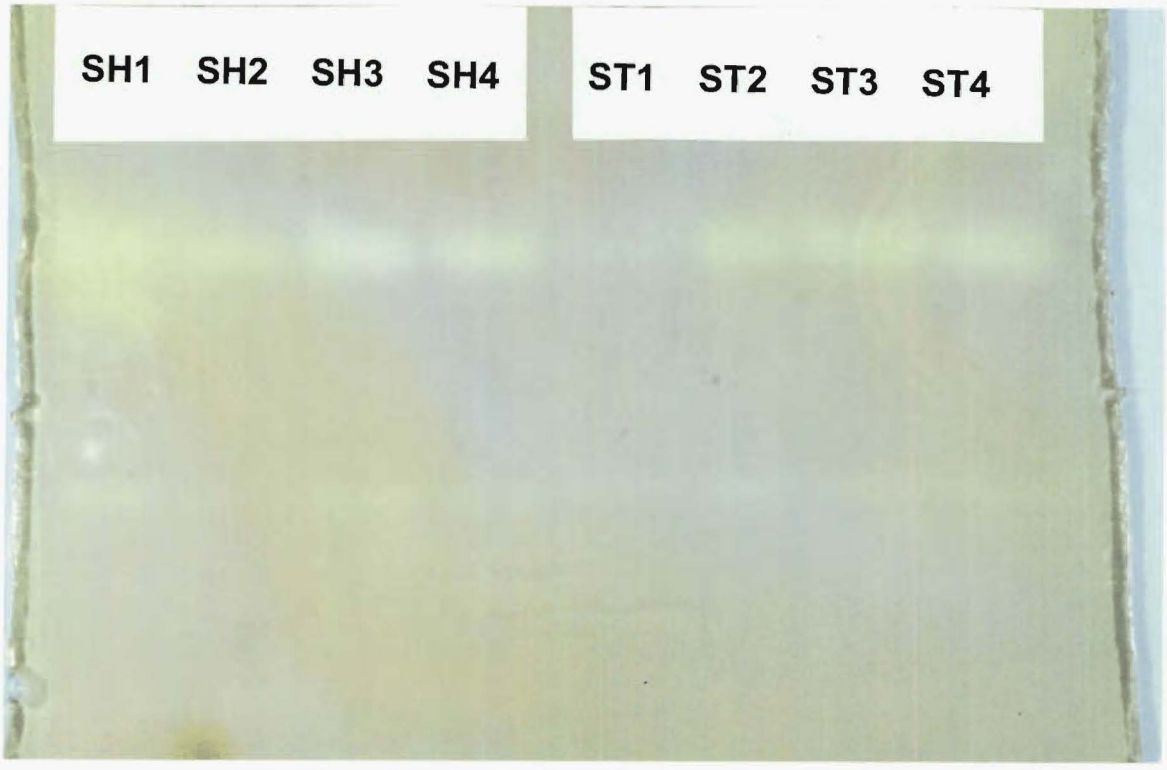


Figure 36. Superoxide Dismutase activity in cultures of *P. expansum* grown in YE medium.

4.4 SUMMARY.

Some differences were seen in the activities of primary and NADPH generating enzymes in *P. expansum* grown in both types of media, however not every pathway appeared to show a response to agitation.

ME medium reducing sugar levels were low and fed into primary metabolism slowly. The static culture was very slow to utilise these sugars. By comparison, the YE reducing sugar levels were high, and therefore could not be considered limiting to flux through metabolism. While the static utilisation lagged over the first 24 h, the amount of reducing sugar used matched that of the shake culture by the end of the 96h.

Glucokinase was assayed as a monitor of glycolytic activity and did not appear to be affected by aeration in ME medium. Slight increases in shake activities were noted in the YE medium, although these were not considered significant. Therefore the same amounts of active enzyme were available for both aeration conditions in this pathway. Sugar transport was proposed as a potential limitation to activity of this pathway as an effect of lower ATP generation, through reduced electron transport chain activity through the lack of terminal O_2. Fructokinase levels were very low and similar for shake and static cultures in ME medium. In YE medium, the activity of this enzyme oscillated in both shake and static culture, and this was matched by the fluctuations in activity of mannitol dehydrogenase.

Glucose-6-P dehydrogenase activity was higher in shake cultures grown in ME medium between at 48-72 hours, and it is probably due to the presence of two extra isozymes on native PAGE gels at this time. The presence of different isozymes provides a possible means for regulation of this enzyme. Measurement of Michaelis Menten constants showed these to be lower for glucose-6-P and NADPH in the shake cultures than the static ones, indicating a greater affinity for these substrates in the former. A number of cellular metabolites were also studied as potential inhibitors of glucose-6-P dehydrogenase in ME culture extracts. 6-Phosphogluconate and NADPH inhibited this enzyme significantly, and it is proposed that since these are the reaction products that it is likely that they inhibit both competitively and allosterically.

Glutamine, ammonium, ATP, AMP, and erythrose-4-P were also good inhibitors and it is likely these also inhibit allosterically, binding to another site on the enzyme. Erythrose-4-P is a metabolite further down the pentose phosphate pathway and therefore feedback inhibition is likely to be occurring. Omission of Mg^{2+} from the assay to determine if an ATP-Mg^{2+} interaction was occurring showed no significant differences since the errors overlapped on both media. Only the 96h ME static culture showed any differences, which possibly arose from low endogenous Mg^{2+} levels. This is another potential mechanism for regulation in this medium in particular, which was not supplemented with trace elements solution. The somewhat 'false' *in vitro* assay component quantities would not reflect the *in vivo* situation.

6-Phosphogluconate dehydrogenase activities were lower than the glucose-6-P dehydrogenase. An equilibrium is possibly maintained between the levels of 6-PG and glucose-6-P by the activity of the second enzyme of the pathway. A specific glucose-6-P level would be maintained especially if glycolysis was favoured. This possibility is more favourable for the ME medium cultures, but not those grown in YE medium since there would be a higher flux to 6-phosphogluconate generated by the G6PD. YE G6PD and 6PGD activities were quite different since 6PGD activities were increasing. More metabolites would pass through the pathway due to a higher substrate concentration, the metabolites could then be balanced through the equilibrium mentioned before.

Mannitol dehydrogenase was at very low levels in the ME medium, indicating that the mannitol cycle was either not operating or that polyol metabolism was not significant. Fructokinase activities were similar to the mannitol dehydrogenase activities in the static cultures in this medium, but the shake cultures were quite different. YE activities were different to the ME activities. Mannitol dehydrogenase activities were increased in the shake cultures over the first 48 hours, whereas the static cultures became more active after 48 hours. The oscillations in mannitol dehydrogenase activities matched those of fructokinase. It is likely that the mannitol cycle would make only limited contributions to the cellular NADPH pool and would be secondary to effects produced by the pentose phosphate shunt if relative enzyme activity levels are considered.

The enzyme assay for malic enzyme resulted in the ME cultures having greater activity than the YE cultures, which is a reversal of the trend seen in other assays. Shake activity was present and peaked at 48 hours, then decreased to negligible. Static activity was lower but present till later, at 72 hours. The pattern of activity for this enzyme in ME medium resembled that of NAD-isocitrate dehydrogenase, which is not surprising considering the close relationship of malic enzyme to the TCA cycle. Enzyme assays showed activities in YE medium to be almost non-existent, and therefore the ability to generate NADPH using this enzyme would be insignificant. It is likely that malic enzyme activity in YE cultures was inhibited since activity was detected on isozyme gels. The same isozymes were present in all cultures and therefore differences in activity cannot be attributed to differing affinities or quantities of isozymes. Instead, an explanation for regulation could be that allosteric modification of the enzyme could be occurring, and that the inhibitor may have been removed by the electrophoresis process. Other workers have suggested that this enzyme is regulated by substrate availability, since it has to compete against the TCA cycle for metabolites. The similarity of shape in the malt extract medium, particularly the static cultures, indicates that the low substrate concentration of the medium was limiting to both malic enzyme and the TCA cycle. This is also represented in the higher activities of the shake culture, since the flow of metabolites through metabolism would be driven by the availability of the terminal electron acceptor O_2. If the substrate concentration was limiting in the YE cultures this would not affect the level of available catalytic activity, and this is considerably reduced in these cultures.

The levels of NADP-ICDH activity were at a reasonable level, however these are not sufficiently high to be the sole means for NADPH generation in the cell. Static ME levels showed a constant increase over time, whereas the shake activities levelled off late in the culture. In both media the static activities were higher than shake at 96 hours. Increasing levels of activity were observed in the YE cultures over time as well, although both shake and static levelled off after 72 hours. The static culture activities were significantly greater than shake at 72 and 96 hours. This was not reflected in the isozyme gels, which showed a single band to be present throughout both shake and static cultures. It is possible that the differences in activity for this

enzyme are related to fatty acid synthesis, which is regulated without the control of this enzyme, but which affects the flux of metabolites to it, and is regulated in part by the compartmentalised supply of NADPH from this enzyme. Fatty acid biosynthesis is considered with the activities of this and other enzymes in the Concluding Discussion (Chapter 8).

The NAD^+ form of isocitrate dehydrogenase was present at low levels in ME medium. The TCA cycle appeared to be more prevalent in early (up to 48 hours) shake cultures, clearly as a response to agitation. The static activities increased gradually over time but did not increase to a point where this pathway could be considered significant. The YE activity results were quite different. Both shake and static cultures peaked early then decreased over the culture period, although shake cultures showed an increase at 96 hours. The lack of terminal O_2 had less effect on the static cultures than in ME medium, probably because of the greater substrate concentrations, and the increased ability of the organism to utilise metabolites through a number of different pathways. Included in this is the feeding and bleeding of the TCA cycle by processes such as transamination and the utilisation of acetyl CoA in fatty acid biosynthesis.

CHAPTER 5

THE EFFECT OF AGITATION ON POLYKETIDE BIOSYNTHESIS IN *PENICILLIUM EXPANSUM*.

5.1 PATULIN BIOSYNTHESIS: A MODEL POLYKETIDE PATHWAY.

5.1.1 Introduction

Patulin was discovered by Birkinshaw *et al.* in 1943, and was believed to be synthesised in a similar manner to penicillic acid. Patulin is a very intricate molecule, possessing a functional group on every carbon. It has been shown to inhibit the growth of both gram positive and negative bacteria, but its potent carcinogenicity and teratogenicity prevents it use as an antibiotic (Zamir, 1980).

Patulin (4-hydroxy-4H-furo(3,2C)pyran-2(6HO)-one, $C_7H_4O_6$) is a tetraketide mycotoxin produced by a number of fungal species of the genera *Penicillium* and *Aspergillus*, including *P. claviforme, P. equinum, P. novae-zeelandae, P. lapidosum, P. cyclopium, P. roquefortii, A. clavatus, A. giganteus, A. terreus*, and *Byssochlamys nivea* (*Gymnoascus* spp., an ascomycete). Patulin production is usually associated with the species *P.expansum* (blue mould of apples found naturally in soil) and *P.urticae* (*P. patulum*) (Harrison, 1989). It has been referred to as clavacin, clavatin, claviformin, expansin, leucopin, myocoin C, penicidin and tercidin. Patulin is a β-unsaturated lactone; a colourless, crystalline compound, MW 154, and a melting point of 110-112 °C. It is stable at acid pH at temperatures of up to 125 °C, and unstable in alkali. Lovett and Peeler (1973) showed that as pH increased the time required to reduce patulin concentrations decreased for all temperatures studied in the range of 105-125 °C. As a hemiacetal compound it is able to undergo esterification to form benzoate, cinnamate and monoacetate, it is able to react as a simple carbonyl compound after ring opening at the hemiacetal function, and can form compounds

such as a semicarbazone, oxime, and phenylhydrazone (used as a TLC detection spray). (McKinley and Carlton, 1991)

A new reaction of patulin was demonstrated in studies by Rao and Reddy (1989) which attempted to modify the patulin structure to lose the carcinogenic activity. An unusual product called 4-hydroxy(2*H*)pyran-2-one was formed from reaction with sodium hypoiodite and CH_2N_2 methylation, causing the loss of the 2 carbons that were part of the lactone ring.

Various synthetic syntheses of patulin have been achieved. Bennett *et al.* (1991) and Gill *et al.* (1988) synthesised patulin and neopatulin from carbohydrate precursors. Patulin was produced from arabinose through the formation of a protected ketone, followed by a Wittig condensation, acid catalysed cyclisation, then dehydration and deprotection. Neopatulin was formed from lyxose by a similar method. Boukouvalas and Maltais (1995) synthesised patulin in 6 steps from benzyloxyacetaldehyde and 2-(*tert*-butyldimethylsiloxy)-4-[(*tert*-butyldimethylsiloxy)-methyl]furan.

Hopkins (1943) trialled patulin as a potential cure for the common cold since it had been shown to possess bacteriostatic activity, and was stable in serum and pus. 59 % of treated cases recovered from their infections within 48 h compared to 9.4 % in the control group, with no ill effects observed. Boyd (1944) did a similar study using a nasal douche and claimed to observe dramatic and beneficial effects against severe colds, and suggested this was due to decongestive and possibly local anaesthetic properties.

Patulin can be inactivated through its reaction with sulfhydryl-containing amino acids or proteins, as well as SO_2, and vitamin B_1. Cysteine, thioglycollic acid, glutathione, dimercaptoethanol readily react with patulin at a pH as low as 2.3. Its toxicity has been reduced after reaction with enzymes containing sulfhydryls such as urease, aldolase, lactic and alcohol dehydrogenases. Fremy *et al.* (1995) demonstrated that patulin concentrations in laboratory wastes could be eliminated by ammoniation (NH_3 and heat), and oxidation by potassium permanganate under both acidic and alkaline conditions. Ascorbic acid achieved only 36 % degradation after 44 hours incubation with patulin.

Patulin has been isolated from foods infected with mould, in particular apples and apple products such as cider and juice, but also in pears, peaches, apricots, bananas, pineapples and grapes. Moulded foods such as bread, unroasted coffee beans, commercial tomato paste have also been shown to contain patulin, as have pecans, flour, cereals, legumes, apricots, crab apples, persimmons, meat, poultry feed, swiss cheese, and cheddar cheese. However, patulin has not been associated with a natural disease outbreak in humans or animals (such as Turkey X which is caused by aflatoxins).

Ingestion of foods containing patulin has been shown to affect people through gastritis with ulceration and enteritis, gastrointestinal tract lesions, hyperemia and fluid distention. Various animal studies have been performed to determine whether patulin had any carcinogenicity or teratogenicity. 2 mg/mL was embryocidal in mice and chickens. Mutagenic effects were seen in *Bacillus subtilis* and *Escherichia coli*, but not in *S. cerevisiae*. *In vitro*, conflicting results indicate some mutagenicity in human hepatocytes in one study but not in others. In rats, sarcomas were detected at the site of injection. (McKinley and Carlton, 1991).

Patulin has been shown to inhibit cell and/ or nuclear cell division, but there are conflicting obervations as to the nature of its action. It had a deleterious action on *Allium cepa* and *Lepidium sativum* nuclei and chromosomes, with some debate over whether the spindle mechanism is affected, since sulfhydryl compounds such as glutathione, cysteine and mercaptoethanol could prevent the mitostatic effect, but it is also possible that patulin reacts with sufhydryl groups of contractile fibres (Singh, 1967).

Patulin has been responsible for the inhibition of aerobic respiration in a number of systems: bacterial, fungal, guinea pig kidneys and brains. It affects the semipermeability of cell membranes, possibly by either damaging the plasma membrane or altering the transport mechanisms into the cell, although no leakage of metabolites has been noted. Potassium ion uptake by erythrocytes was affected, as was glucose uptake by fungal mycelium, and in toad urinary bladder the Na-ATPase system as affected. The electron transport enzymes succinate dehydrogenase and succinate oxidase were inhibited in *Claviceps purpurea*, but NADH oxidase,

cytochrome *c* reductase, and cytochrome oxidase were relatively insensitive to it. ATPase in human erythrocytes was reported to be inhibited by patulin as well. (Singh, 1967, McKinley and Carlton, 1991). The exact sites and toxicity mechanisms of patulin are not known and it has been proposed that a modified form of patulin may be the toxic agent. Included in the hypotheses for its action is the inactivation of sulfhydryl groups in enzyme active sites, however patulin was slow to bind with cysteine and did not react with glyceraldehyde-3-phosphate dehydrogenase, an enzyme with sulfhydryl groups in its active site. The inhibition of growth of anaerobic bacteria indicates that the site of action in the inhibition of respiration could be prior to the terminal electron transport chain of aerobic respiration. (Singh, 1967; McKinley and Carlton, 1991).

Riley and Showker (1991) showed patulin to be involved in a series of events including lipid peroxidation, abrupt calcium influx, extensive membrane blubbing and total LDH concentration changes in cultured renal cells, each of these events representing a step in the loss of structural integrity of the plasma membrane. Inhibition of the Na^+/K^+ pump in these cells was not the sole cause of the changed Na^+ and K^+ content of the cells resulting in increased hyperpolarisation. Patulin selectively altered the K^+ efflux relative to Na^+ and the transient hyperpolarisation effect of patulin was thought to be due to this K^+ efflux. Critical membrane sufhydryls were oxidised by patulin. Patulin-induced lipid peroxidation was not confined to just one cell type (i.e. renal and muscle cells), and may be a general property of patulin cytotoxicity.

Miura *et al.* (1993) studied the effects of patulin on protein prenylation on mouse FM3A cells, and showed that at a concentration of 290 μM patulin inhibited farnesyl:protein transferase in a cell free system. This enzyme is responsible for post-translational modification of proteins through the formation of thioether-linked prenyl groups. The incorporation of [^{3}H]mevalonate into proteins and non-saponifiable lipids was inhibited at a patulin concentration of 7 μM.

Many studies have been concerned with patulin levels in apple juice and its detection. The WHO level for patulin in apple juices is 50 μg per litre. In a survey of Australian apple juices, Watkins *et al.* (1990) showed that of 29 Victorian brand names

investigated, 65 % were contaminated with patulin, and attributed this to the use of rotten apples in the juicing process. Patulin is relatively stable in apple juice, and is not destroyed by treatments such as 80 °C for 10-20 minutes. Other studies have shown that pasteurisation treatments such as HTST (high temperature short time) at 60-90 °C for 10 seconds, and batch pasteurisation at 90 °C for 10 minutes (Harrison, 1989) reduced patulin levels, however treaments such as these can cause changes to the flavour, colour and aroma of the juice. Sydenham *et al.* (1995) showed an initial water treatment step transferred the patulin from the apples used for processing from the solid phase to the aqueous phase, reducing the concentration of patulin in the final product. Fermentation of apple juice by Saccharomyces spp decreased the toxin concentrations by degradation into non-volatile water soluble substances (Harrison, 1989). Filtration through, or agitation with, charcoal also removed patulin from juice.

Wilson and Nuovo (1973), using qualitative TLC analysis, showed that patulin was produced by all 60 isolates of *P. expansum* Link studied at the level of at least 10 μg/mL, with 16 isolates in the range of 9-18 mg/L and one having a range of 126-146 mg/L.

Paster *et al.* (1995) studied patulin production by *P. expansum* grown on pears and apples at different temperatures and modified atmospheres. Patulin production was totally inhibited when fungi were stored in 3 % CO_2/ 2 % O_2 at 25 °C. Increasing the O_2 concentration resulted in increasing amounts of patulin production. The conditions for controlling patulin production varied between different strains. These workers also showed that the pathogenicity of *P. expansum* and patulin production are not necessarily correlated. The preservatives, sorbate, benzoate, and SO_2 decreased the growth of *B. nivea* over a range of temperatures. Patulin was produced in the range of 12-37 °C, and SO_2 had the greatest effect on biomass and patulin production by this organism (Roland and Beuchat, 1984).

Okeke *et al.* (1993) demonstrated that fungal extracts containing patulin could inhibit the growth of 3 important rice phytopathogens *Pyricularia oryzae, Drechslera oryzae* and *Gerlachia oryzae.* They also identifed a number of new patulin producing organisms including: *Eupenicillium javanicum, P. cyaneum, P. diversum, P.*

duclauxii, P. echinulatum, P. glabrum, P. lignorum, Alternaria tenuissima, Chaetmium atrobrunneum, Scopulariopsis flava and a *Stemphylium* sp.

Other studies have identifed conditions under which patulin is produced by fungal cultures. Jimenez *et al.* (1991) showed that stationary dark cultures of *P.griseofulvum* grown on yeast extract-sucrose medium produced more patulin than shaken or fermentor cultures, with the best temperature for production being 28 °C. Patulin degradation was accelerated by vitamin C and SO_2, and also by alcoholic fermentation and ionizing radiation. Temperature and water activity of the cultures affected production also. Shaking produced 2-3 mm pellets under these conditions.

Nip and Chu (1977) used replacement cultures to maximise the production of patulin by *P. patulum*, and in doing so provided a method for the production of [^{14}C] patulin from [1-^{14}C] acetate. Cultures were static grown on PDB until resting and then transferred onto a glucose-acetate-salts medium.

Escoula (1975a) showed that patulin was produced by *B. nivea*, *B. fulva*, and *Paecilomyces varioti*, isolated from silage produced patulin grown on enriched Czapeks medium (with glucose and yeast extract) at 26 °C. Further studies by this author on *B. nivea* (1975b) showed that addition of propionic and formic acids decreased mould growth and production of patulin and another mycotoxin byssochlamic acid.

The production of patulin has been used with enzyme assays (the API ZYM system) and griseofulvin analyses to differentiate isolates of *Penicillium griseofulvum* Dierckx. Two groups amongst the strains were distinguished which were unable to be differentiated by morphological or cultural characteristics (Jimenez *et al.*, 1990). Boysen *et al.* (1996) used patulin production (as well as the formation of other metabolites, and DNA sequence alignments, RAPD analyses, and 18s and 28s genes) to classify *Penicillium carneum* and *P. paneum* as separate species after originally being grouped with *P. roqueforti* varieties.

5.1.2 The Patulin Biosynthetic Pathway.

The biosynthesis of patulin has been studied closely and a number of different biogenetic theories proposed.

The net reaction for patulin biosynthesis is as follows (Gaucher *et al.*, 1981):

$$\text{Acetyl CoA} + 3\ \text{Malonyl CoA} + 3\ \text{NADPH} + 3\ H^+ + 3\ O_2 \rightarrow$$

$$\text{Patulin} + 4\ \text{CoASH} + 4\ H_2O + 3\ NADP^+ + 4\ CO_2$$

From glucose:

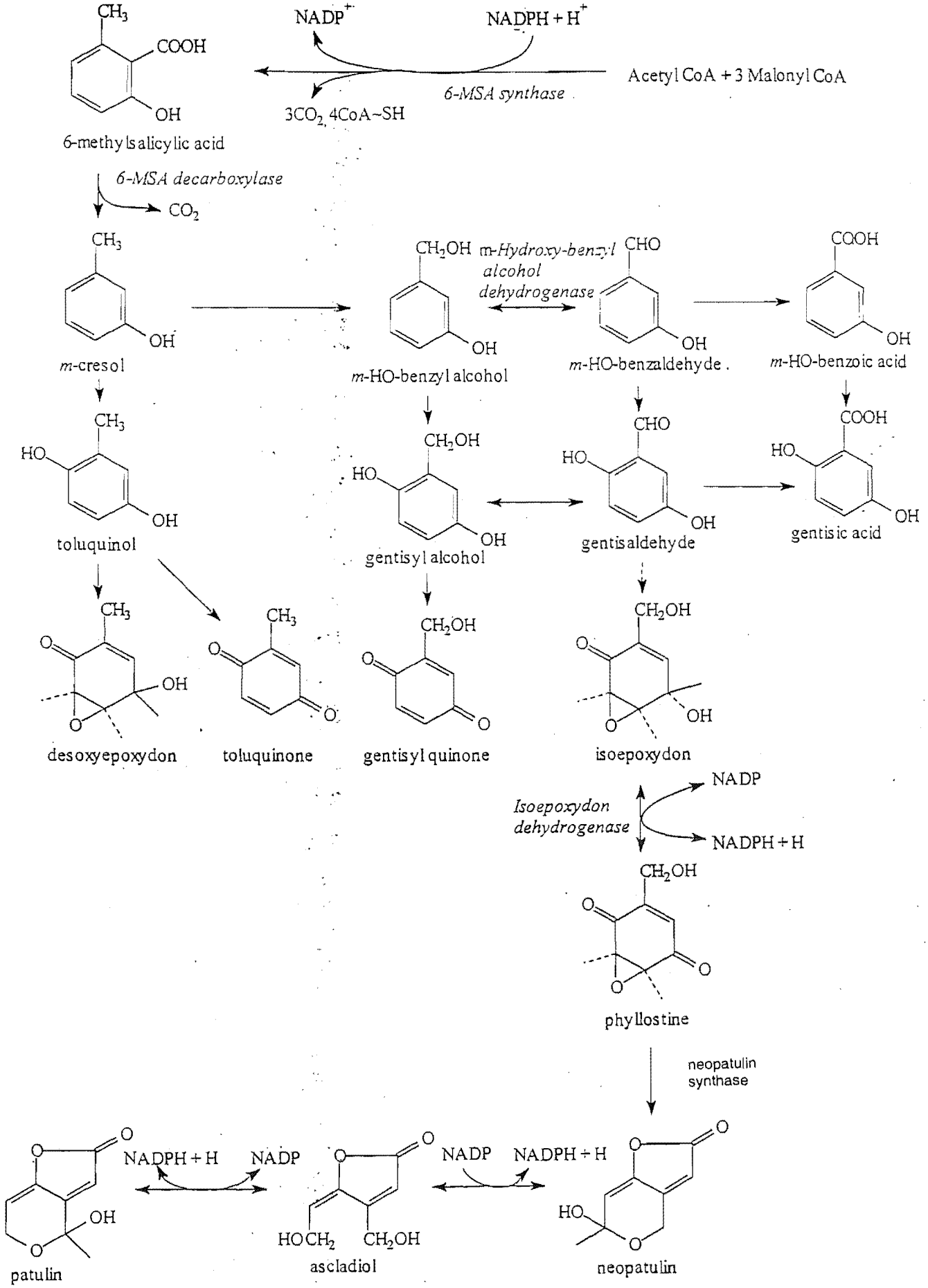
$$2\ \text{Glucose} + 5\ ½\ O_2 + 12\ \text{ADP} + 12\ P_i \rightarrow \text{Patulin} + 5\ CO_2 + 21\ H_2O + 12\ \text{ATP}$$

The biosynthesis of patulin requires only glucose and O_2, and has the potential of producing a net yield of ATP under aerobic conditions.

Figure 37 shows the patulin biosynthetic pathway. Polyketide biosynthesis starts with a series of Claisen condensations of malonyl CoA and acetyl CoA, in which a repeating C_2 unit from malonyl CoA is added onto acetyl CoA, with the loss of the remaining C as CO_2. The first major intermediate in the patulin biosynthetic pathway is 6-methyl salicyclic acid (6-MSA), which is produced by a multienzyme complex called 6-MSA synthase in a similar manner to fatty acid biosynthesis. The synthesis of fatty acids involves seven repetitions of the same chain of events. The diversity of polyketide structures reflects incomplete cycles. The programming of the product structure is largely dictated by the specificity of each enzyme component in the process (Jordan and Spencer, 1993). Bassett and Tanenbaum (1960) were able to show that a cell-free preparation of *P. patulum* could convert acetyl CoA and 6-MSA into patulin, in a process that required 'active' acetate.

The 6-MSA synthase complex features two sulfhydryl groups labelled Sp (peripheral) and Sc (central) which provide the attachment points for acetyl CoA (at Sp for the release of CoA) and malonyl CoA (at Sc), the reaction of which is inhibited by *N*-ethyl maleimide and iodoacetamide. Decarboxylation of the malonyl group and condensation with the acetyl group occur, producing an acetoacetyl unit on the Sc.

Figure 37. The patulin biosynthetic pathway (Neway and Gaucher, 1982).



The acetoacetyl group is transferred to Sp and another malonyl (CoA) group attaches to Sc and the reaction continues until a C_6 polyketo group is generated on Sc, which then undergoes reduction using NADPH resulting in dehydration to form a *cis*-double bond. Another 2 malonyl CoA-mediated attachments occur, followed by an internal aldol reaction and dehydration to give 6-MSA bound as an acyl derivative to the multienzyme complex. (Zamir, 1980)

A 4'-phosphopantotheine residue has been identified as part of the enzyme which acts as an acyl carrier flexible arm, similar to that on FAS (Zamir, 1980). Monooxygenase generates gentisaldehyde using O_2, NADPH, is stimulated by ATP and inhibited by Fe^{2+} chelators.

Turner and Aldridge (1971) showed that *m*-cresol, as well as toluquinol, gentisyl alcohol and gentisaldehyde, could be incorporated into patulin using deuterium-labelled precursors and MS analysis. Feeding of putative precursors has been done, resulting in the formation of unusual metabolites. If propionyl CoA was used instead of acetyl CoA by an *in vitro* preparation of purified 6-MSA synthase, the result was the production of 6-ethyl-salicylic acid. Studies with deutero-toluquinol showed that this compound was not incorporated into patulin production, indicating that toluquinol is a by-product of the patulin pathway and not an intermediate; an unknown optically active compound (a 'forced metabolite') named deoxyepoxydon was produced. (Zamir, 1980).

Dimroth *et al.* (1970) purified 6-MSA synthase 100-fold from submerged cultures of *P. patulum*. The 6-MSA synthase complex co-migrated with FAS in gels performed at purification, indicating this enzyme shared properties with FAS. In the absence of NADPH, triacetic acid lactone was produced at a rate only 10 % that of 6-MSA production, indicating that in the synthesis of 6-MSA, reduction occurs at the triacetic acid level before the final condensation with malonyl CoA. The difference in rates for the production of these 2 compounds was proposed to be due to the covalent linkages of the respective substrates to the sulfhydryl groups during synthesis. Another study on triacetic acid lactone synthesis by the same organism confirmed that the omission of NADPH resulted in this compound being formed at levels 3x higher than when

NADPH was included, and that 6-MSA synthesis occurs 12x faster than TAL formation, in molar terms this means that the system synthesised 6-MSA 4x faster than TAL (Scott *et al.*, 1971). These authors proposed that TAL could be involved in 6-MSA biosynthesis since dehydration, condensation with malonyl CoA and further dehydration would give 6-MSA based on evidence they had obtained using an *in vitro* model which generated diketo-octenoic acid in situ, which could undergo dehydrative aromatisation to form 6-MSA.

Bu'Lock and Powell (1965) studied the synthesis of 6-MSA and the metabolites of *P. urticae* with respect to glucose metabolism, distinguishing clear tropophase-idiophase growth by the reduction in respiration at the end of tropophase, and the reduction of withdrawl of TCA intermediates for synthetic reactions. They postulated that the stimulus for the onset of 6-MSA production would be the accumulation of acetyl CoA or more probably of malonyl CoA. Bu'Lock (1966) indicated that patulin synthesis could actually be inhibited by 6-MSA possibly by a specific feedback inhibition on the 6-MSA synthase enzyme, or by a general inhibitory effect of salicylates. He suggested that the organism had a mechanism for regulating the uptake of 6-MSA from the medium, since studies showed that after 30 minutes of uptake of this compound from the medium, it was expelled.

An important study by Forrester and Gaucher (1972a) used kinetic pulse-labelling experiments with acetate and eight other secondary metabolites fed to submerged cultures of *P. urticae*. Patulin was shown to be readily converted from 6-MSA, acetate, *m*-cresol, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde and gentisaldehyde, indicating the preferred route for patulin biosynthesis. Toluquinol and gentisyl alcohol were not incorporated. Patulin was not converted to any other secondary metabolites.

Phyllostine was identified as an intermediate on the patulin biosynthetic pathway of *P. urticae* by Sekiguchi and Gaucher (1978) by comparing and contrasting results of feeding experiments using the J1, J2, S11, S15 mutants and the parent strain, in which a number of previously unidentified compounds accumulated. When phyllostine was fed to mutants with an intact post-gentisaldehyde portion of the pathway, patulin was

produced. Phyllostine is formed by a monooxygenase from gentisaldehyde via gentisquinone by an internal redox reaction.

Isoepoxydon was identified in a similar manner by Sekiguchi and Gaucher in 1979 (a). It was shown to be an obligatory precursor to phyllostine, and these two compounds were interconverted by an alcohol dehydrogenase-type reaction by isoepoxydon dehydrogenase.

The *P. urticae* mutant S11 accumulated isopatulin (neopatulin) in small amounts, and could be produced by cells immobilised in acrylamide which were fed phyllostine (Sekiguchi *et al.*, 1979). The P3 mutant (blocked between phyllostine and isopatulin) was fed isopatulin and was able to produce patulin, indicating this compound was indeed a precursor to patulin.

The final reaction on the patulin biosynthetic pathway was determined by Sekiguchi *et al.* (1983). Transformations of neopatulin and ascladiol were studied in cell-free extracts of the mutants J1, S11 and S15. A requirement for NADPH was determined for the step between neopatulin and ascladiol, and its absence or replacement by $NADP^+$ inhibited the conversion. Non-enzymic conversion was also observed, resulting in the formation of (Z)-ascladiol as well as (E)-ascladiol. The catalytic mechanism is believed to involve sulfhydryl attachment to the activated C6 of neopatulin.

5.1.3 6-MSA Synthase: Polyketide Synthase Gene Structure.

The 6-MSA synthase gene has been the focus of attention of several workers. This enzyme catalyses the first seven reactions in the biosynthesis of patulin and has been noted for its similarity to the gene that gives rise to fatty acid synthase (FAS). These differ at the cyclisation and enoyl reduction reactions, which are unique to 6-MSA synthase and FAS respectively (Beck *et al.*, 1990). The *P. urticae* PKS (consisting of 4 α-subunits) and vertebrate FAS (consisting of 2 α-subunits), are composed of similar multifunctional peptides, and both enzymes produce free carboxylic acid products. In comparison, the fungal FAS contained two different multifunctional polypeptides, and both enzymes produced free carboxylic acid products (Wang *et al.*, 1991).

Beck *et al.* (1990) showed that in *P. patulum* (*P.urticae*), this enzyme was a homomultimer of a single, multifunctional protein unit induced at the transcriptional level at the end of logarithmic growth. The identity of the 6-MSA synthase gene was established by three independent criteria. Firstly, the gene product specifically reacted with anti-6-MSA antibodies. Secondly, the nucleotide sequence correlated at several analysed positions with the 6-MSA synthase amino acid sequence. Finally, the isolated DNA exhibited distinct, although in restricted portions, sequence similarities that determined for other known PKSs. Preparation of a homogeneous enzyme preparation showed the subunit molecular mass to be 188 kDa. Using immunological screening of a genomic *P. patulum* DNA expression library, the 6-MSA synthase gene was identified as a 5322 base pair open reading frame (ORF), encoding a protein of 1774 amino acids and a molecular mass of 190 731 Da.

Most polyketide synthases share a maximum of four different active sites which include: acyl and malonyl transferases; the acyl carrier protein (panetheine binding site); and the 2-oxoacyl synthase (or the 'peripheral' sulfhydryl group). These have also been identified in the FAS of yeasts and animals. A specific set of component enzymes, which operate in a distinct order, define the identity of the end product of each different synthase. Beck *et al.* (1990) had also located 3 *P. patulum* introns, which had similar characteristics.

Wang *et al.* (1991) were also able to clone a portion of the 6-MSA synthase gene from this organism. They obtained a 7.7 kb genomic DNA fragment, which contained the 3' terminus using a 41-mer mixed oligonucleotide probe which was based on cyanogen bromide cleavage of the pure protein. Sequence analysis showed a 1866 base pair ORF without introns, which corresponded to the amino acids of the carboxy terminus of the enzyme, followed by a putative trancriptional termination – polyadenylation signal. Using the sequences characteristic of FAS functional demains, they showed that the 3' terminus was preceded by β-ketoreductase (KR) domain, then a putative acyl carrier protein (ACP) domain. This enzyme showed better homology with the vertebrate FAS rather than that from yeast or *P. urticae*. The KR and ACP domains gave a degree of homology to vertebrate FAS of 67 % and 50 % respectively.

Robinson (1991) reviewed the structure and function of polyketide synthases in antibiotic synthesis. The fatty acid synthase and polyketide synthase of monesin A-producing *Streptomyces* are similar, but the latter must be more programmed since during the assembly of the monesin triene intermediate, it must select in a specific order the building blocks from acetyl, propionyl and butyryl residues, incorporate the correct chemical functionality during each chain extension step, and establish the correct relative and absolute configuration at each new chiral centre and double bond. PKS enzymes are programmed to assemble one out of a large number of theoretically possible reduced polyketide chains.

Fatty acid synthases fall into two categories. The type II FASs are plant and bacterial in origin, and can be readily separated into discrete globular proteins, which catalyse the consecutive steps of the FA synthase cycle. These do not need to form into aggregates. The type I FASs are found in yeasts and mammals, and are present on one multifunctional polypeptide chain. The catalytic sites are believed to be arranged in a series of globular connected domains.

The structures of PKS complexes are much harder to establish because they are expressed at low levels and the assays used are not sufficiently sensitive. The genes provided a much easier approach since they can usually be found close to the antibiotic resistance gene. Sequencing of the PKS genes for actinorhodin, granaticin, and tetracenomycin showed that discrete polypeptide chains were encoded. Monensin PKS did not have a sequence homologous to the putative keto-reductases of FASs, and this is consistent with the lack of reductive synthesis during catalysis by this PKS.

However, it is also possible that the folding of the polyketide chain can affect the identity of the peptide produced. Granaticin and tetracenomycin PKSs are similar at the protein level, but generate polyketide chains of different lengths and different folding patterns. All of these *Streptomyces* PKSs resemble bacterial type II FASs, rather than the large multifunctional polypeptides.

The fungal PKSs tend to resemble the type I FASs. *Saccharopolyspora erythraea* produces erythromycin and the PKS involved in its biosynthesis could be divided into 9 separate portions which had active site sequences similar to the consituent catalyitc

activities of known FASs and PKSs. The sequences in the assembly of this macrolide matched the order of ORFs encoded on the multifunctional polypeptide: i.e. AT (acyl transferase) – acyl carrier protein (ACP) – ketoreductase (KR) – AT - β-ketoacyl synthase (KS) – ACP – KR – AT – KS.

The enzymology, mechanism and molecular programming in FASs and PKSs which give rise to tetraketides was reviewed by Jordan and Spencer (1993). Molecular programming is defined as the regulation of the reaction pathway. 6-MSA synthase uses the non-repetitive programming process, where reduction occurs only once during the three reaction cycles catalysed by the enzyme, at the C_6 polyketide intermediate stage. This reduction is a key step in the reaction, and if the enzyme is starved of NADPH during the reaction, triacetic acid lactone is formed. There is no obvious reason why a single reduction occurs during 6-MSA biosynthesis. It is not certain whether this dehydration occurs at the C_6 alcohol, or if left until the cyclisation step, although the former has greater favour.

Several different experimental approaches have been taken in establishing the reactions of 6-MSA synthase (Jordan and Spencer, 1993). Protein studies using *P. patulum* 6-MSA synthase showed there was an absence of thioesterase. The 6-MSA synthase was not inactivated by PMSF, which would react with the active site serine of the thioesterase, raising the interesting possibility that the product may finally be released from the enzyme by a novel mechanism. Another study used the cross-linking agent 1,3-dibromopropan-2-one. 6-MSA synthase formed dimers with a molecular weight of 360 000, suggesting that this may operate as a functional dimer. The cross-linking was prevented by acetyl CoA and malonyl CoA.

Another approach was to incorporate other intermediates into 6-MSA. Acetoacetyl CoA was used as the starter unit to study the steric course of reaction mechanisms with (*R*) and (*S*) malonyl CoA derivatives. A chain termination reaction occurred in which the C6 polyketide intermediate was released from the enzyme as the triacetic acid lactone, a reaction normally occurring in the absence of NADPH at a rate approximately 5 % that of the normal reaction. It is possible that the catalyic residues from the reductase (or the dehydratase) may be involved in the triacetic acid lactone release mechanism both in 6-MSA synthase and FASs.

The stereochemistry was also considered for the intermediate alcohol, since the absolute configuration of the C_6 hydroxy intermediate is likely to be of crucial importance to the molecular programming. The FAS configuration of the alcohol intermediate is (*R*), and mechanistic constriants would arise in the dehydration reaction to form the *cis*-double bond required for aromatic ring formation. However, it is not known whether a (*R*) or (S) is involved in the 6-MSA synthase reaction.

The role of acyl carrier protein in regulation was also considered. The intermediate-ACP substrate forms the basis of the reaction with the enzyme. The disposition of the polyketide intermediate in an extended or cyclical conformation is a result of a specific reaction which ACP may play a role in determining a reaction pathway.

The stereochemical course of the biosynthesis is determined in part by the elimination of H atoms from the 2- and 4- positions of the C_6 polyketide (H_{Si} and H_{Re}), and the Claisen condensations tend to occur with inversion of configuration. This would impose mechanistic restrictions on subsequent reactions. Other considerations included the specificity of the enzyme activities for the ACP- intermediates, the steric course of the reductase and dehydratase reactions, and the role played by the polyketide-ACP. There may also be a role for the enzyme in the stabilisation of larger polyketide intermediates, possibly by enolisation.

5.1.4 Regulation of Patulin Biosynthesis.

Grootwassink and Gaucher (1980) showed that the biosynthesis of patulin involved the *de novo* formation of enzymes at the onset of secondary metabolism. Their experiments showed that changes to the medium N content affected the timing of the appearance of *m*-hydroxylbenzyl alcohol dehydrogenase. The formation of this enzyme and 6-MSA synthase were affected by addition of the protein synthesis inhibitor, cycloheximide or actinomycin, therefore indicating that biosynthesis could not be initiated by pre-formed latent proteins. This study was also important because it achieved complete separation of the replicatory growth phase and idiophase in batch cultures of *P. urticae*.

Gaucher *et al.* (1981) used mutants of *P. urticae* to demonstrate the effects on patulin biosynthesis. The mutant in the first pathway enzyme only produced the second and

fourth enzymes at 10% of the normal levels. Addition of pathway metabolites (*m*-cresol or patulin) resulted in normal enzyme levels, indicating that coordinate induction of enzymes after the lesion by these metabolites occurred.

There are a number of factors which can affect the biosynthesis of patulin. The first group are extrinsic limitations, and include: a glucose concentration of less than 5-6 % in the initial medium; O_2 at levels below 10-20 % of saturation; and trace metal ions (Gaucher *et al.*, 1981).

Intrinsic limitations form the second group. Neway and Gaucher (1981) removed all the extrinsic limitations on patulin production by *P. urticae* in order to identify the first/ principal intrinsic limitation. Given optimum conditions for aerobic glucose catabolism, primary metabolite precursors should not be limiting, therefore the first pathway enzyme whose cellular contents fall constitutes the site of the initial intrinsic limitation (substrates accumulate and products become limiting). This enzyme was 6-MSA, and the decrease in concentration occurred in both the YEGB medium, containing trace metals and buffering agents, and the unbuffered Yeast Extract Glucose medium. Other enzymes involved in patulin biosynthesis also decreased, but over a significantly larger time scale.

5.1.4.1 Nitrogen.

Grootwassink and Gaucher's work (1980) indicated that the medium nitrate concentration was responsible for the appearance (at low concentrations) or disappearance (when N was added back to the medium) of secondary biosynthesis enzymes. Rollins and Gaucher (1994) showed that ammonium was responsible for the repression of patulin biosynthesis in *P. urticae*. They performed experiments with a range of ammonium concentrations (5-30 mM) in shake cultures to monitor the effect of this on the formation of metabolites and the levels of marker enzymes. Patulin biosynthesis was initiated when the concentration of ammonium was decreased from 30 mM to 4 mM. 6-MSA synthase appeared at an ammonium concentration of 2.5 mM. A discussion of nitrogen regulatory mechanisms is given in Section 2.5.2.

Addition of ammonium ions after the onset of derepression had little effect on regulation and would only affect the replenishment of pools of secondary metabolism mRNAs and enzymes depleted by turnover.

This work also showed that the ammonium concentration affected the production of intracellular proteinases in *P. urticae*. See below in Section 5.1.4.3.

5.1.4.2 Manganese.

The effect of supplementation of culture medium with manganese for the production of patulin by *P. urticae* was shown by Scott *et al.* (1984). Deficiencies in this metal ion resulted in the accumulation of high levels of 6-MSA, which appeared later in the culture. There were also changes in the morphology of this fungus – the mycelial mat became granular and friable rather than compact and filamentous. Pellets were dense and tightly interwoven with little peripheral growth, with reduced internodal distance between hyphal branches.

These workers established that a dose-response relationship between Mn^{2+} and patulin production occurred between 1.5 – 100 μM Mn^{2+} if this organism was grown on a complex nitrogen source. The appearance of patulin pathway metabolites were delayed and in reduced concentrations. Cultures were also less pigmented when grown in Mn-deficient medium. Interestingly, the cultures lacking Fe were not pigmented at all. Deficiencies in copper, zinc and iron had no effect on patulin biosynthesis. The manganese effect was deemed unique to secondary biosynthesis since there was no growth inhibition.

Further work by Scott and Gaucher (1986) showed that the cellular levels of Mn changed during the transition to patulin production. There were three distinct phases of cellular Mn content. The first from 15-22h reflected a steady decrease in levels at the end of the active growth phase. From 22-26h, steady uptake of Mn into cells occurred (phase II). Phase three was a steady state period in which the newly attained levels remained constant. It was suggested that a Mn-specific transportation system may exist. The presence of energy uncouplers increased uptake rather than decreased it, indicating that metabolic energy might be necessary to Mn efflux rather than influx. Work with protein synthesis inhibitors did not affect uptake, indicating protein

involved in this function was pre-existing. Previous workers had noted that 6-MSA could be involved in metal ion uptake. 6-MSA could possibly be unable to re-enter the cell for conversion unless neutralised by a metal ion.

The site of Mn control of patulin biosynthesis was shown to be at the coordinate induction of pathway enzyme transcription (Scott *et al.*, 1996). This ion was implied in the coordination of derepression of all but the first enzyme. The effect of Mn on primary metabolism enzymes was studied as well. Only glucose-6-phosphate dehydrogenase (G6PD) of the Pentose Phosphate shunt showed a marked and reproducible decrease in the Mn-deficient cultures. The difference in enzyme activity after 24h could have been due to the significant amount of NADPH required for patulin biosynthesis. Since patulin production would not occur in the Mn-deficient cultures, there would be less of a requirement for enhanced G6PD activity. It was proposed that direct metal ion inhibition of primary metabolic enzymes such as G6PD would lower the cellular $NADP^+$/NADPH ratio to favour polyketide biosynthesis over fatty acid biosynthesis. However, there was no evidence of a gross disruption of primary metabolism affecting secondary metabolism. Other studies reviewed by these authors suggest that there is a possibility that Mn is required for a mechanism that controls age-specific events at the level of transcription.

5.1.4.3 Intracellular Proteases.

The levels of intracellular proteinase were monitored in *P. urticae* in relation to 6-MSA synthase (Lam *et al.*, 1988). This enzyme has been shown to be more labile than other enzymes and was able to be stabilised *in vitro* using NADPH (the cofactor), acetyl CoA and malonyl CoA (substrates), dithiothreitol (a reducing agent) and phenylmethylsulfonyl fluoride (proteinase inhibtor). This indicated that proteolysis and conformational integrity of the enzyme control the duration of antibiotic synthesis *in vivo*.

Rollins and Gaucher (1990) showed that intracellular proteinase activity in cell extracts increased 2-fold during storage. The proteinases isolated showed properties consistent with a possible role in general protein turnover. This indicated a possible

role in the supply of new amino acids for the *de novo* production of antibiotic biosynthetic enzymes.

The regulation of these proteinases in *P. urticae* by medium ammonium concentrations was shown by Rollins and Gaucher (1994). The patulin biosynthetic enzymes tended to lose activity upon proteolysis, but the proteinases still functioned. However, the repression by nitrogen was much greater for the patulin enzymes than for the proteinases.

5.1.5 Analysis of patulin.

Patulin has also been detected by a number of methods. The heptafluorobutyrate derivative of patulin was detected by capillary gas chromatography by Tarter and Scott (1972). Ehman and Gaucher (1977) used capillary GC with a Gas Chrom Q column using a temperature programme to detect the phenols that make up the patulin biosynthetic pathway, derivatised with trimethyl silyl. Thin layer (and paper) chromatography has also been widely used. Aqueous phenyl hydrazine solution (Scott *et al.*, 1972), dinitrophenyl hydrazine (Woodhead and Walker, 1975) and 3-methyl-2-benzothiazolinone-hydrazone hydrazine (MBTH) (Sekiguchi and Gaucher, 1977; Leuenberger, 1978; and Gimeno, 1979) have been used as sprays. Sekiguchi and Gaucher (1978) performed radiochromatography of ^{14}C-labelled patulin precursors running plates in chloroform: ethyl acetate: ethyl ether or chloroform: acetic acid solutions. Radioactive spots visualised by UV were scraped into a scintillation solution and quantified. Direct and indirect ELISA assay of patulin was also possible through the reaction of polyclonal antibodies against patulin hemi-glutarate (McElroy and Weiss, 1993), the immunogenicity of patulin was increased by linking to BSA.

A patulin-soaked filter paper disc was placed on a plate culture of *Bacillis brevis* in a disc diffusion assay. The resulting zone of growth inhibition indicated the relative concentration of the mycotoxin (Madhyastha *et al.*, 1994).

The most common method for quantification of patulin is reversed-phase HPLC. Commonly, a C_{18} column or similar has been used, but a number of different solvent systems have been used for the mobile phase. Stray (1978) and Moller and Josefsson (1980) used water as the mobile phase, while Prieta *et al.* (1993, 1994) and Rovira *et*

al. (1993) used 99+1 water and tetrahydrofuran (THF). Bartolome *et al.* (1994) used a water-acetonitrile or a water-methanol gradient, the former was used by Scott *et al.* (1994) as well and was the method used in this work.

Extraction of patulin has also been achieved by a number of means. Solvent extraction using ethyl acetate is the most common method and Prieta *et al.* (1993, 1994) developed this using dialysis in this solvent to extract patulin from apple juice. A Sep-Pak SiO_2 cartridge was used to clean up extracts further by Rovira *et al.* (1993). Solid phase extraction was used by Leuenberger *et al.* (1978), in which apple juice was applied to an Extrelut column and subsequently eluted with toluene-ethyl acetate mixture.

5.2 CITRININ

The second polyketide produced by the *P. expansum* strain used in this thesis is citrinin. Citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid) is a pentaketide polyketide produced by some species of *Aspergilli* and *Penicillia* (*A. flavus*, *A. terreus*-Reddy and Bernt, 1991; *A. candidus*, *P. citrinum*-Steyn, 1991; and *P. expansum*-Woodhead and Walker, 1975). It was produced by 46 % of the *P. expansum* strains tested on a laboratory medium and 73 % of these strains were isolated from decaying apples (Vinas *et al.*, 1993). It exhibits colour changes with a change in pH, for example at pH 4.6 it is lemon yellow and at pH 9.9 it is cherry red. As a strong acid is soluble in alcohol, dioxane and dilute alkali, but is insoluble in water (Reddy and Bernt, 1991).

As an antibiotic, citrinin exhibited $^1/_{600}$ of the bacteriostatic power of penicillin, however there was host toxicity. Citrinin is an important nephrotoxic mycotoxin. It has been responsible for the disruption of renal function through swelling, necrosis and desquamation of the tubular epithelium in the proximal convulted tubules. Studies have also shown citrinin to be embryocidal, fetotoxic and mildly teratogenic, as well as having a depressant effect on respiration and the cardiovascular system, causing vasodilation and bronchoconstriction. It is commonly implicated with

ochratoxin A in nephrotoxic diseases, including endemic Balkan nephropathy (Reddy and Bernt, 1991).

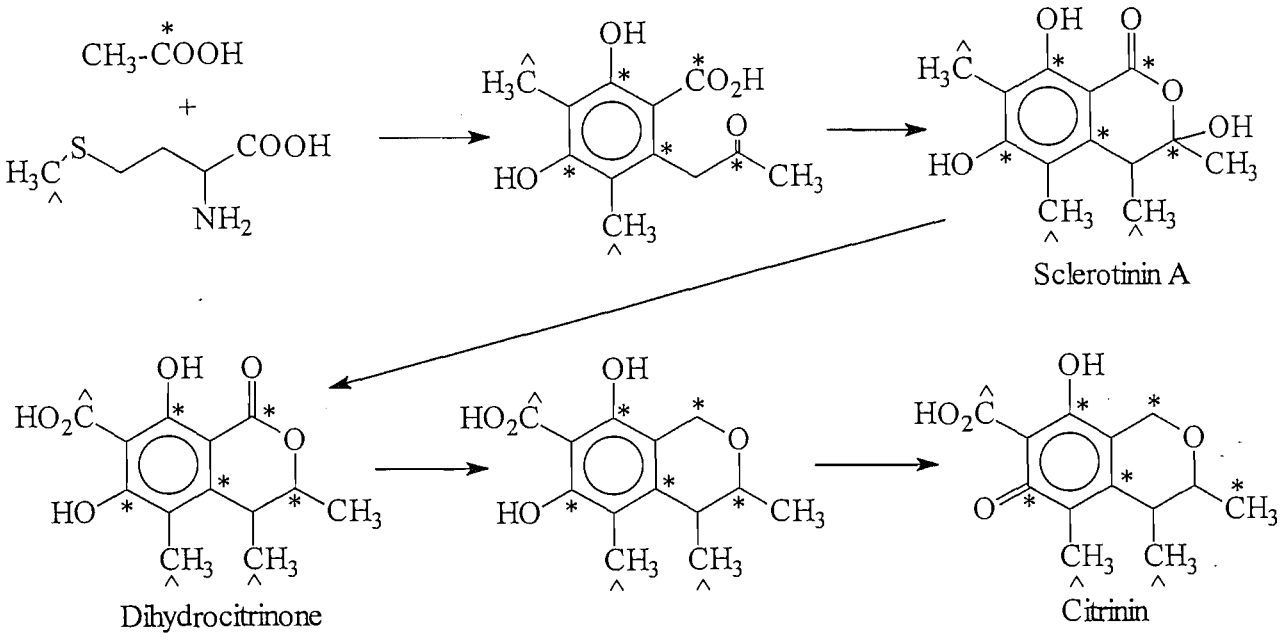


Figure 38. Possible pathway for citrinin biosynthesis (Steyn, 1992).

Figure 38 shows a possible pathway for the biosynthesis of citrinin. Citrinin is derived from acetate to form the pentaketide, and the carbon methylation at C_4, C_5 and C_7 (including the carboxylic acid group) is provided by methyl methionine (methionine on which an extra methyl group is substituted onto the sulfhydryl S atom).

Blanc *et al.* (1995) studied citrinin production by *Monascus ruber* grown in various liquid and solid culture media. The best yields in liquid media were found when the organism was grown on a yeast extract sucrose (16 %) medium under static conditions, however the best yields were achieved on solid media. Production was not favoured when methionine or urea were used as the N source. Both a low C/N ratio and the N source were important for citrinin synthesis. Citrinin production was

favoured when nitrites or nitrates were used, whereas amino acids (especially glutamic acid) favoured red pigment production. Limitation of O_2 concentrations could also reduce citrinin production. The production of citrinin and the red pigment were regulated by the acetyl CoA flux, and exogenous acetate followed the polyketide pathway.

A similar conclusion drawn by Betina *et al.* (1973) showed that citrinin production by *P. notatum* Westling S-52 was diphasic, with the first phase occurring in tropophase and the second in idiophase. The utilisation of acetyl CoA by primary metabolism regulated citrinin production in this organism, since N limitation resulted in lower TCA activities, leaving acetyl CoA available for lipogenesis and citrinin synthesis. The free and esterified fatty acid levels changed when the C:N ratio was changed. The total lipid levels and citrinin tended peak at opposite times to each other.

Citrinin has been detected by a number of methods including TLC, with detection by a BF_3-methanol spray (Scott *et al.*, 1972) or oxalic acid-impregnated plates (Marti *et al.*, 1978), or by spraying with aluminium chloride, which resulted in a blue fluorescence (Gimeno, 1979). Xiao *et al.* (1995) used the coupling of the C_8 hydroxyl group to iodoacetate and 1,1'-carbonyldiimide in an ELISA assay. HPLC has been used with a number of solvent systems. A C_{18} column was used by (Blanc *et al.*, 1995) with a methanol-water gradient elution. Whilst Marti *et al.* (1978) used phosphoric acid-methanol or phosphoric acid-acetonitrile with a reversed phase column and fluorescence detection (325-386 nm excitation and 451nm emission).

5.3 OBJECTIVES

Initial work in these studies focused on qualitative analysis of the polyketides produced by *Penicillium expansum* using paper chromatography of ethyl acetate extracts of culture medium. This was followed up with quantitative analysis of the same extracts using reversed phase high performance liquid chromatography (HPLC) to determine the levels of patulin produced by this organism under different aeration and nutritional conditions.

Repeated attempts at assaying *m*-hydroxybenzyl alcohol dehydrogenase in mycelial extracts were unsuccessful, and while this information would have been a very useful indication of secondary metabolic activity in this organism, this aspect of work was abandoned. Similarly, the first enzyme of the patulin biosynthetic pathway, 6-methyl salicylic acid (6-MSA) synthase, was not studied routinely because of the prohibitive cost of radio-labelled malonyl CoA.

The specific production parameter was used to describe the difference in metabolite accumulation according to the biomass (in terms of dry weight) of the producer culture. This was calculated by peak area/g dry weight. This was necessary because in some cases there were large amounts of metabolites in the culture medium compared to other times or conditions, but this was a consequence of the producer organism being of a greater biomass at that particular time, and therefore a lower specific production. This parameter would have been useful in comparison to pathway enzyme activities, had they been monitored.

Objectives for this section of work included:

(1) Qualitative assessment of the polyketide secondary metabolites produced by *P. expansum* produced under shake and static conditions under the differing nutrient conditions provided by ME and YEGB media.

(2) Quantification of the concentration of patulin produced by *P. expansum* under shake and static conditions under the differing nutrient conditions provided by ME and YEGB media.

5.4 MATERIALS AND METHODS.

5.4.1 Culture conditions.

These have already been given in Chapter 3. Shake and static cultures of *P. expansum* were grown in YE and ME medium for these studies.

5.4.2 Secondary metabolite extraction.

Fungal mycelium from liquid cultures was removed by filtration through Miracloth. The culture medium was then filtered using Whatman no. 114 filter paper. Samples (25 mL) of culture filtrate were taken and acidified with 5 mL 0.1 M-HCl. This was then extracted with two 30 mL aliquots of ethyl acetate. The combined top layers were evaporated to dryness in a rotary evaporator at 35 °C. The residue was then taken up in 95 % v/v ethanol for further analysis.

5.4.3 Qualitative analysis of culture filtrate extracts using paper chromatography.

Culture filtrate extracts prepared as above were spotted onto Whatman No. 1 or 3 MM chromatography paper. Standards of 6-MSA, patulin and citrinin dissolved in 95 %v/v ethanol were used in initial studies, and later these were extended to include *m*-hydroxylbenzyl alcohol, *m*-hydroxybenzaldehyde, toluquinol, and gentisic acid. The chromatograms were developed in either 2 % v/v acetic acid (HAc) or 125:72:3 v/v/v benzene:acetic acid: water (BzAc), prepared by mixing 2:2:1 v/v/v benzene: acetic acid: water together, allowing the phases to separate to take the top layer for use as the chromatography solvent. Two solvent systems were used since some compounds running together in one were separated from each other in the other.

Chromatograms were run in duplicate since two different sprays were used for metabolite detection. Chromatograms were air dried prior to further treatment.

3-Methyl-2-benzothiazolinone-hydrazone hydrazine (MBTH) was used to visualise patulin according to Prieta *et al.* (1992); chromatograms (coded PAT) were sprayed with a 0.5 % v/v solution and oven dried 15 minutes at 130 °C. Patulin appeared as a yellow-orange spot in visible light, and which fluoresced orange under UV light. Diazotized *p*-nitroaniline (Brentamine fast red GG salt) was used to detect other phenolic metabolites. Chromatograms were first dipped in saturated sodium carbonate solution and air dried then counter-sprayed with 0.5 % w/v solution of Brentamine. The dry chromatograms then oven dried to develop coloured spots.

Results for the standards using the two solvent and spray systems are given in Table 4.

5.4.4 Spectrophotometric analysis of extracts.

Culture filtrate extracts prepared as outlined in Section 5.4.2 were diluted 50 x in 95 % ethanol and analysed using a Hewlett Packard 8452A diode array spectrophotometer against a 95 % ethanol blank. It was necessary to dilute samples to 1:50 (v/v) for spectrophotometric analysis and in some cases 1:2500 (v/v) dilutions were required. Although this method was not used for quantitative analysis, it was used to corroborate the results from the paper chromatography analysis. Standards of patulin, 6-MSA, *m*-hydroxybenzyl alcohol, *m*-hydroxybenzaldehyde, toluquinol, gentisic acid and citrinin were prepared in 95 % ethanol and spectral maxima obtained for each to aid in identifying unknown spots on the paper chromatograms. Information gained on the standards is presented in Table 4 in the Results (Section 5.5)

5.4.5 Reversed phase high performance liquid chromatography analysis of patulin.

Samples (10 µL) of were injected onto an Econosil C18 5U reversed phase column (250 mm x 4.6 mm, Alltech) fitted with a 2 µm frit and All-Guard Cartridge system (Alltech), and eluted with a water (containing 0.5 % v/v acetic acid) acetonitrile gradient based on that used by Scott *et al.* (1984). A linear gradient of 0-35 % acetonitrile was run over 30 minutes during which time patulin was monitored at 276

nm. Five wavelengths were monitored simultaneously: 276nm (patulin), 254nm (*m*-hydroxybenzyl alcohol), 295nm (toluquinol), 305nm (6-MSA), 330nm (citrinin); retention times are given in the Results Section 5.5.3 in Table 5.

The HPLC system was a Shimadzu model LC-10AT with diode array detector (SPD-M10A), and Shimadzu Class M10A LC Workstation software.

Patulin was quantified using 10 μg/mL patulin dissolved in 95 % ethanol as an external standard

5.5 RESULTS.

Table 4. Paper chromatography of standards of metabolites possibly accumulated by *P. expansum*.

Compound	λ_{max}	UV colour	MBTH reaction	Brentamine (Na_2CO_3)	R_f BzAc	R_f HAc
Patulin	276	-	orange	brown	0.77	0.87
6-MSA	242 308	blue	purple* (0.5-2 µg)	purple	1.00	0.74
m-OH benzyl alcohol	256	-	purple* (0.5-2 µg)	crimson	0.16	0.77
m-OH benzaldehyde	276	blue	violet/ cream centre* (1-5 µg)	brown (white)	0.67	0.73
toluquinol	292	purple (non-fluoro)	pink	brown-purple	0.20	0.73
Gentisic acid	236	light blue	brown* (0.5-2 µg)	brown	0.15	0.64
Citrinin	330	yellow	purple	orange	1.00	0.59

*represents colour determined by Sekiguchi and Gaucher (1977), number ranges indicate detection limit (per spot).

The BzAc solvent system was not used for the detection of patulin using the MBTH spray. The paper chromatography results were purely qualitative and no information regarding relative quantities could be gathered from the results. Despite this, it should be noted that the extraction method was consistent for each sample, and the same extracts were used for HPLC analysis later. In any chromatographic spray system there is a lower limit of detection, and therefore small concentrations of a given metabolite in the culture media may not be detected by a particular spray system. Therefore spots not detected in this work may have been below the detection limits given in Sekiguchi and Gaucher (1977), shown in Table 4.

5.5.1 YEGB medium results.

5.5.1.1 Paper Chromatography: Qualitative secondary metabolite analysis.

Figures 39, 40 and 41 represent the results typically obtained for paper chromatography of culture filtrate extracts of *P. expansum* grown in YEGB medium.

Citrinin was absent from the metabolites extracted from this medium, possible reasons for this are discussed later (Section 5.6).

6-MSA and toluquinol, both which yield purple spots with the Brentamine spray, ran to similar places in the HAC solvent, however in BzAc 6-MSA ran with the solvent front and toluquinol had an R_f of approximately 0.2. On this basis, the metabolite appearing at a R_f near 0.74 in HAc and 0.2 in BHH was tentatively identified as toluquinol or toluquinone (if the quinone was formed this would explain the reaction to form a hydrazone with MBTH). It is reasonable to assume that 6-MSA would be the main candidate for this compound since it is the first main intermediate of the patulin biosynthetic pathway, however the R_f data suggests otherwise. It is not inconceivable that toluquinol (or toluquinone) could be produced by *P. expansum* since in *P. urticae* it is derived from *m*-cresol, a patulin pathway intermediate, probably through the action of a mixed-function oxidase (MFO) (Neway and Gaucher, 1981).

The chromatograms suggest that toluquinol was produced in shake cultures after 24 hours. Its presence in static cultures occurred sporadically, and therefore was considered more of a shake culture product than a static one. The production of *m*-hydroxybenzyl alcohol was contrary to this. This compound was present only in static 72 and 96 hour old cultures, and is on the 'main' patulin production pathway. The dehydrogenase reaction which catalyses the conversion of this alcohol to *m*-hydroxy-benzaldehyde, is favoured thermodynamically in the reverse direction since the reverse reaction rate compared to the forward rate is approximately 6:1 (Forrester and Gaucher, 1975). This could explain why the aldehyde was not detected on any of the chromatograms.

Toluquinol and *m*-hydroxybenzyl alcohol are both derived from *m*-cresol in *P. urticae* (Neway and Gaucher, 1981), differing by the position of hydroxylation of this compound (toluquinol has an *ortho* hydroxylation on the aromatic ring, whereas *m*-hydroxybenzyl alcohol undergoes hydroxylation at the methyl group). Both reactions are believed to use O_2 and NADPH for the hydroxylation, therefore any preference for one to be produced rather than the other under specific culture conditions would not

be a reflection of the availability of either of these co-substrates. Instead, a likely explanation could be that the MFO catalysing the conversion to toluquinol becomes active in the presence of O_2, in the shake cultures, drawing flux from the alcohol-producing enzyme. Accumulation of *m*-hydroxybenzyl alcohol would therefore occur when O_2 is present in lower concentrations since there would be greater levels of it produced from *m*-cresol. The potential effects of this branch point on the patulin concentration are discussed later with the patulin quantitation results (Section 5.6). Another branchpoint for the off-take of metabolic precursors is a possible pathway parallel to patulin, producing isomeric forms of intermediates starting at desoxyepoxydon (produced from toluquinol, and parallel with isoepoxydon). This would produce a series of related metabolites (Priest and Light, 1989). This is referred to later in this section as a possible source of the "yellow" compound.

Priest and Light (1989) postulated that a parallel pathway to patulin branching at *m*-cresol may exist, based on the ability of particulate fractions of *P. patulum* to catalyse the epoxidation of toluquinol. They suggested that the proportion of metabolite flow into the parallel pathway would be determined by the relative activities of the *m*-cresol ring and methyl hydrolase activities. The methyl group hydroxylation has a lower K_m (5 μM) for *m*-cresol than the ring hydroxylation (20 μM), and the former must occur first for patulin biosynthesis.

Another potential metabolite accumulated by this culture was gentisic acid. It was produced on only one occasion in shake cultures older than 24 hours and in static cultures, and generally was not present in the other replicate cultures studied. This is considered with the ME medium results in Section 5.6.

The presence of patulin in YEGB culture filtrates was assessed qualitatively using the MBTH reagent. Patulin appeared as an orange spot at R_f 0.87 on HAc chromatograms, and was present at 48 hours in the shake culture and before 72 hours in the static cultures (Figure 41). Little information about the relative levels of patulin could be obtained using this technique. These results will be discussed with the quantitative results using HPLC in Section 5.6.

Other compounds that reacted with the MBTH reagent included citrinin, toluquinol and a yellow compound, which ran at an R_f similar to patulin in the HAC solvent. This compound was present in cultures earlier than patulin, often produced with it but not usually in the late stages of the culture in this medium. Several of the patulin pathway metabolites were available and tested for a similar reaction, including those shown in Table 4. None of these formed a yellow product and several possibilities were considered as to its identity.

It was considered that the compound could be a destabilised patulin product occurring in culture media above pH 6. Patulin undergoes hydrolysis above pH 6 since it is a lactone, and degradation increases with a higher pH (Jimenez *et al.*, 1990), and it was postulated that the yellow compound may be related to this. This hypothesis was later discounted. The YEGB medium was pH 6.5 at the start of the culture (Chapter 6), undergoing a steady decrease as the culture progressed, as the fungus acidified the medium. The same occurred in the ME culture medium, which started at pH 5.9, becoming even more acidic than the buffered YEGB medium.

Another possible explanation was that the 'yellow compound' was a metabolite on the citrinin biosynthetic pathway; however since citrinin did not appear to be produced under these conditions this was unlikely. Yet another possibility was that patulin may have formed adducts with other compounds (e.g. sulfhydryls on proteins) in the culture medium. This was also discounted since a review of literature indicated that other workers had also found this compound. Sekiguchi and Gaucher (1979(b)) proposed that the 'yellow compounds' were more than likely late pathway metabolites situated between the epoxyquinones (phyllostine, isoepoxydon and patulin). They also showed that phyllostine could be directed towards the 'yellow' compounds depending on whether cell-free extract was supplemented with NADPH. *In situ* levels of $NADP^+$ were sufficient to allow conversion of isoepoxydon to phyllostine to the 'yellow' compounds, indicating that phyllostine was the immediate precursor to these. The fact that the J1 and S15 mutants were not able to convert phyllostine to the 'yellow' compounds ruled out the possibility of non-enzymic conversion occurring.

Priest and Light (1989) studied the epoxidation of toluquinol and gentisyl alcohol in *P. patulum* (*P. urticae*). They also found yellow reaction products were formed by reaction with MBTH on TLC of extracts from this organism. TLC analysis of epoxidase action on toluquinol showed a yellow spot after spraying with MBTH and heating. They did not isolate or identify this product, however they suggested its presence was related to the ring opening of desoxphyllostine, and that it was likely to be a compound on a pathway parallel to the patulin pathway involving desoxyneopatulin and desoxyascladiol.

Therefore the identity of the 'yellow compound' accumulated by *P. expansum* in these studies is proposed to be neopatulin or ascladiol, based on the results of the authors reviewed above.

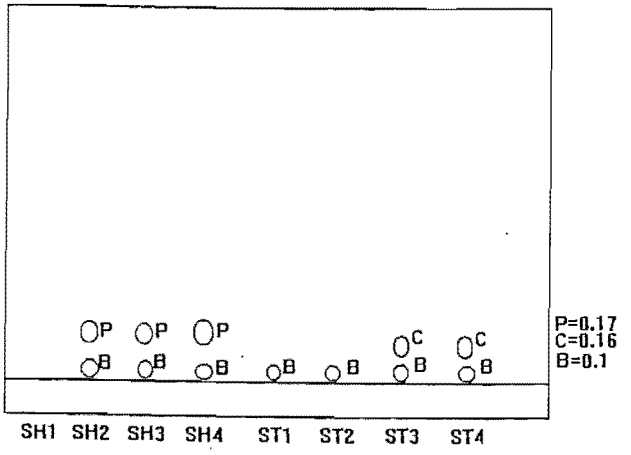


Figure 39. Secondary metabolites of *P. expansum* grown in YEGB medium. Benzene-HAc-H_2O chromatogram.

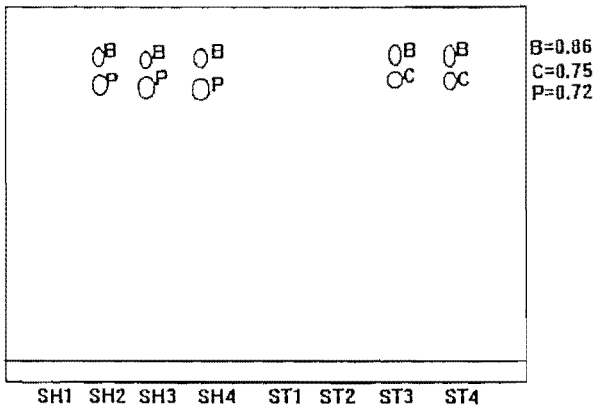


Figure 40. Secondary metabolites of *P. expansum* grown in YEGB medium. 2 % v/v Acetic acid chromatogram.

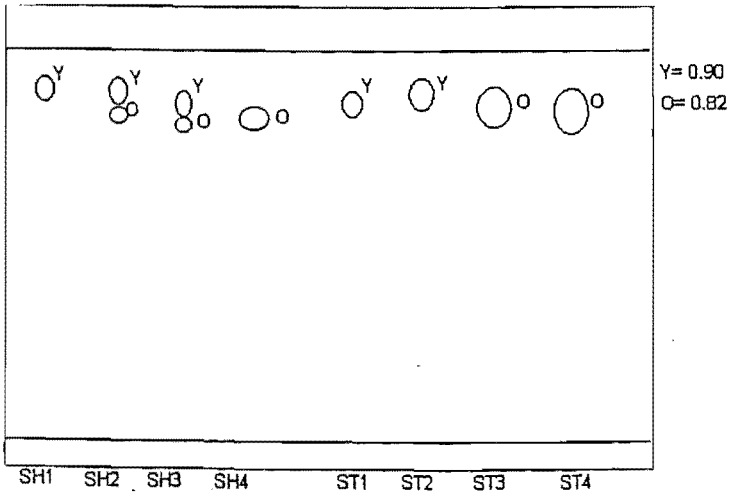


Figure 41. Patulin in YEGB *P. expansum* culture filtrate extracts run in 2 % Acetic acid, using MBTH spray.

5.5.1.2 Spectrophotometric analysis of YEGB culture filtrate extracts.

It can be difficult to establish the presence of one particular compound in a mixture when using spectrophotometric analysis unless peaks can be defined clearly. The wavelengths for each of the polyketide standards are shown in Table 4. Differentiation between 6-MSA and toluquinol proved difficult because of the similar spectral maxima of these compounds (6-MSA λ_{max}= 305 nm; toluquinol λ_{max}= 295 nm). There were also problems associated with identifying these peaks in the presence of patulin because these appeared as an elbow on the main patulin peak (λ_{max}=276 nm).

As indicated in Table 4, *m*-hydroxybenzyl alcohol absorbed at 276 nm, which is the same as patulin. This metabolite was possibly present in shake cultures from 24-96 hours, and more than likely present in static cultures older than 24 hours. The peaks associated with 6-MSA or toluquinol were present in shake extracts only older than 24 hours. Gentisic acid (λ_{max}= 236 nm) was possibly present in a 96 hour old static culture. There was no marked absorbance at 330 nm, suggesting that citrinin was absent from the YEGB cultures.

Based on paper chromatography analysis the conclusions drawn about the spectrum of possible secondary metabolites of *P. expansum* appear to hold for what has been observed with chromatographic analysis. There were two wavelengths at which absorbances not associated with any of the standards. One at 288 nm, which occurred after 24 and 48 h in the YEGB shake cultures but not in static cultures. This did not appear to correlate with the 'yellow spot' of the paper chromatograms. Its λmax suggests that it may be toluquinol (λ_{max}= 295). Another unassigned peak was 264 nm, which was high present in static cultures aged 24 to 72 hours. One possibility for the existence of this peak is that it is situated between 276 nm and 254 nm, the λ_{max} for patulin and *m*-hydroxybenzyl alcohol respectively. If both of these were similar absorbances, the 264 nm peak would be present as a consequence of these compounds.

These results are correlated with HPLC data in Section 5.6, in particular for the determination of the presence of toluquinol and 6-MSA.

5.5.2 ME Medium results.

5.5.2.1 Paper chromatography: qualitative secondary metabolite analysis.

Figures 42, 43 and 44 represent the results of paper chromatography of the culture filtrates produced from *P. expansum* grown on ME medium. The metabolite identified as toluquinol (see Section 5.5.1.1) accumulated throughout shake cultures, but not in static ones whilst 6-MSA appeared to be absent if the toluquinol identification was correct. This reflects what was found in the YEGB medium, although earlier initiation of production was observed in the lower nutrient ME medium. The case for *m*-hydroxybenzyl alcohol was similar; static cultures accumulated this compound throughout the culture period, however there was limited accumulation in shake cultures over 24 hours old.

The conclusions drawn for the YEGB medium regarding these two metabolites would appear to hold also for malt extract medium. The MFO catalysing the conversion of *m*-cresol to toluquinol is possibly activated by increased O_2 availability through agitation, resulting in a branching of the metabolic flux to toluquinol. In this medium, the concurrent production of *m*-hydroxybenzyl alcohol after 24 hours in shake cultures is likely to reflect the nutrient poor status of the organism under both aeration conditions causing secondary metabolism to occur. This is discussed further with the patulin quantitation results in Section 5.6.

Gentisic acid (dihydroxybenzoic acid) sometimes accumulated in 72 and 96 h shake and static cultures. This compound is produced from gentisaldehyde which is a metabolite on the main patulin biosynthetic pathway (Neway and Gaucher 1981) and it represents the most oxidised form of the dihydroxy-substituted ring. The mechanism for its production is unclear. The fact that it accumulated (but not in every culture examined) in late shake and static cultures probably reflects the poor nutritional status of the organism, and that secondary metabolism was well established at this time in both shake and static cultures.

The 'yellow' compound (discussed already in Section 5.5.1.1) was present in almost all of the shake and static cultures from 24 hours in ME medium, indicating that the later section of the patulin pathway was active throughout the culture. This section would appear less affected by oxygenation conditions since phyllostine is produced by dehydrogenation of isoepoxydon, and neopatulin is a rearrangement of phyllostine. A ring opening mechanism, possibly by a monooxygenase, has been suggested to participate in the pathway after gentisaldehyde (Sekiguchi and Gaucher, 1978).

Patulin was detected by the MBTH spray after 24 hours in the shake cultures and after 24 hours in the static cultures grown in ME medium. This indicates that the onset of secondary metabolism occurred earlier in this medium than in YEGB, most probably because the low nutrient concentration in the medium would be depleted faster than in the richer YEGB medium. The quantitative analysis of patulin concentration in this medium is presented in Section 5.5.4.

Citrinin was detected in the ME culture filtrates by both MBTH and Brentamine sprays. Both shake and static cultures produced it on days 3 and 4 in this medium. This would indicate that the synthesis of this compound was not affected by the level of aeration or agitation of the culture. The absence of citrinin in the more nutrient rich YEGB medium leads to the possibility that some form of catabolite repression mechanism may be operating in *P. expansum* in that medium.

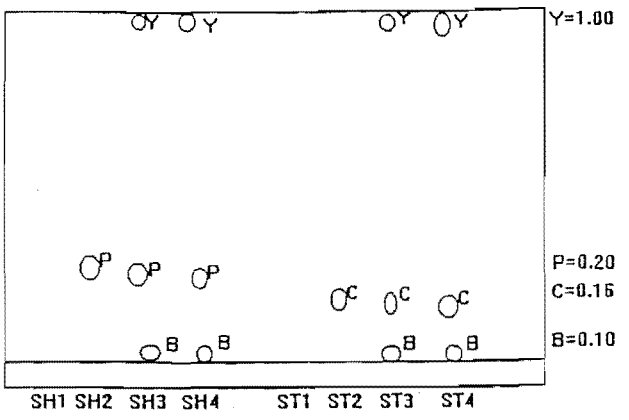


Figure 42. Secondary metabolites of *P. expansum* grown in ME medium. Benzene-HAc-H_2O chromatogram.

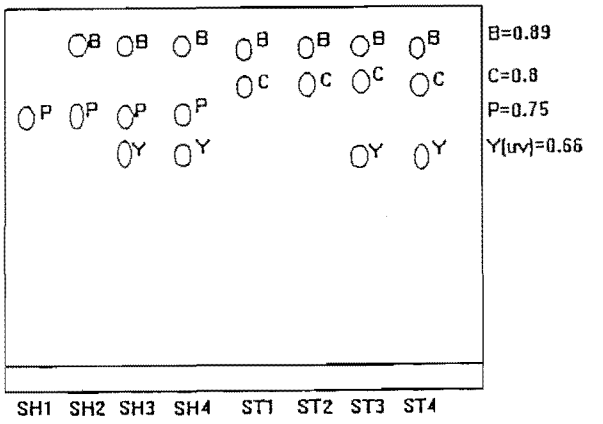


Figure 43. Secondary metabolites of *P. expansum* grown in ME medium. 2 % v/v Acetic acid chromatogram.

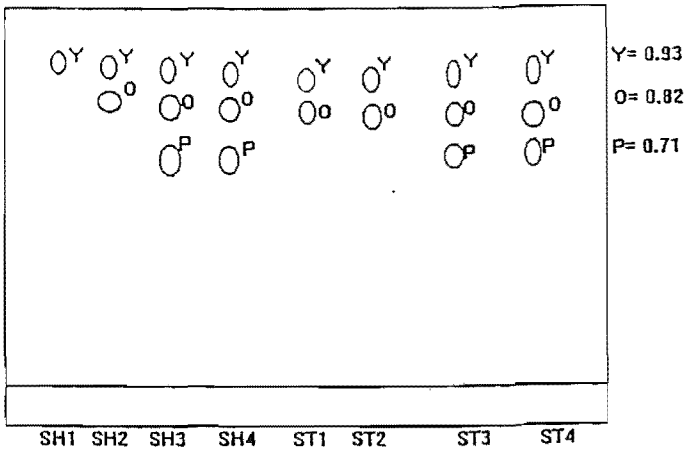


Figure 44. Patulin in ME *P. expansum* culture filtrate extracts run in 2 % Acetic acid, using MBTH spray.

5.5.2.2 Spectrophotometric analysis of ME culture filtrate extracts.

Table 4 summarises the spectral maxima for standards used in determining the presence of polyketide metabolites spectrophotometrically. The absorbance peak/region associated with 6-MSA or toluquinol (as explained in Section 5.5.1.2) was present only in ME shake cultures over all 4 days monitored. *m*-Hydroxybenzyl alcohol appeared to be present in all shake and static cultures but Citrinin was present only in static cultures and after 24 hours. This differs from the paper chromatography results since it would appear to be produced earlier. This difference could be explained by the relative sensitivities of each of these methods, indicating that the spectrophotometric method is possibly more sensitive for the detection of citrinin at 330 nm, than MBTH spraying of chromatograms. Gentisic acid appeared to be present in static culture filtrates at 72 and 96 hours.

Each of the other known compounds monitored appeared to correlate with the paper chromatography results. Once again, absorbance was noted at wavelengths not accounted for by the standards available for this study, in particular at 288 nm and 264 nm. The 288 nm peak was present in all the shake cultures and over the first 48 hours of the static cultures. The 264 nm peak was present only in the first 48 hours of shake ME cultures and in the last 48 hours (i.e. 72-96 hours) in the static cultures.

5.5.3 HPLC analysis of secondary metabolism in *P. expansum*: Qualitative analysis.

The retention times and wavelengths for some of patulin pathway metabolites and citrinin are shown in Table 5.

Analysis of the HPLC chromatograms provided a better means to assign patulin pathway metabolites to a particular wavelength, since compounds with similar λ_{max} could be differentiated on the basis of retention times. This proved to be the case for 6-MSA and toluquinol, which were difficult to distinguish using normal spectrophotometric and paper chromatography analysis, but which eluted at different

times using this RP-HPLC method. Gentisic acid was not monitored using this method.

Table 5. HPLC data for patulin pathway metabolites using RP-HPLC.

Metabolite	Wavelength (nm)	Approximate retention time (minutes)
m-Hydroxybenzyl alcohol	254*	10.5
Toluquinol	295	10.9
Patulin	276	12.4
Citrinin	330	16
6-MSA	305	18

* The elution of *m*-hydroxybenzyl alcohol was usually associated with a peak at 276 nm as well as the 254 nm peak.

The 'yellow' compound may possibly contribute to patulin absorbance if it is a similar compound to patulin. For example, if this compound was generated from toluquinol it would potentially contain one less hydroxyl group, resulting in a different arrangement. The compound would more than likely be more hydrophobic, eluting slightly later than patulin, but may retain many of the factors contributing to absorbance at 276 nm.

Initially, patulin was the main focus of the HPLC analysis, but eventually this was extended to include the other polyketides as the paper chromatography results became more clear. This work was done in a separate series of culture trials, using a different set of extracts to those used for paper chromatography/spectrophotometric analysis. Statistically this is referred to as 'blocking' since there is no way of ensuring the cultures were absolutely identical in their conditions and growth patterns, increasing the probability of variability between culture runs.

Note too that in the Tables that follow (Tables 6-10), the static 24h culture values for specific production of each metabolite appear erroneously large (apparently by an order of magnitude). This can be explained if the dry weight of the mycelium at this time is taken into account since this is an order of magnitude lower than the subsequent days of the culture. Simple division of the peak area by the dry weight therefore results in a high result for these compounds at this time. Because this is an illogical result, the static 24h results should be ignored, and considered only

qualitatively. The comparisons drawn should still be considered qualitative despite the attempt in each table to describe the results in a quantitative manner.

5.5.3.1 YEGB qualitative HPLC results.

The analysis of secondary metabolism provided by this method gave results similar to those obtained using paper chromatography and spectrophotometric analysis, whilst providing clarification of compounds that appeared similar in previous analyses.

6-MSA could be differentiated from toluquinol by HPLC, therefore an indication of when it is likely to be accumulated in cultures can be determined (Table 6). In the YEGB medium 6-MSA appeared to accumulate sporadically in some culture runs and at different times in others. The only day it had not been detected was the 48 h shake culture. There was a tendency for greater concentrations to occur in both shake and (particularly) the static 96 h cultures. This indicates a point in time at which secondary metabolism has become more important in *P. expansum* grown in YEGB medium, where patulin production is produced in the greatest amounts.

Table 6. 6-MSA specific production in YEGB medium.

Time (h)	Shake (peak area/g dry weight) x 10^5	Static (peak area/g dry weight) x 10^5
24	7.9	43.9
48	-	5.1
72	3.4	3.2
96	4.8	21.7

m-Hydroxybenzyl alcohol was present in both shake and static cultures over the 4 day culture period (Table 7). This was in contrast to the paper chromatography results, which showed this compound to be present only in static cultures. A possible explanation for this is in the limits of detection of each method. The chromogenic sprays provide only a qualitative view of what is present. It is possible therefore that the static culture extracts were more likely to have a higher concentration of *m*-hydroxybenzyl alcohol than shake, therefore falling within the limits of detection. However, this is not the case when the relative peak areas are examined in the raw data.

Although in the same magnitude (1 x 10^6), the shake culture peak areas exceed the static culture areas for *m*-hydroxybenzyl alcohol in each run. It should be noted that the HPLC analysis of culture extracts was done several months apart from the paper chromatography/spectrophotometric analysis, leading to 'blocking' of results, and may explain the difference in outcomes between HPLC analysis and paper chromatography/ spectrophotometric analysis.

Table 7. *m*-Hydroxybenzyl alcohol specific production in YEGB medium.

Time (h)	Shake (peak area/g dry weight) x 10^6	Static (peak area/g dry weight) x 10^6
24	6.45	15
48	3.34	2.9
72	1.85	1.1
96	2.43	-

Detection of toluquinol using HPLC resulted in different results to those obtained by paper chromatography/ spectrophotometric analysis (Table 8). Toluquinol did not appear in each of the HPLC analyses of the different culture runs of shake cultures 48 and 72 hours old, and more frequently in the analyses of different culture runs of the 96 hour shake cultures and in all static cultures. However, peak areas on the resulting chromatograms indicated that the YEGB shake culture extracts were more concentrated in toluquinol than the static cultures (not accounting for the dry weights as shown in Table 8). This lends support to the conclusions drawn from the paper/ spectrophotometric results about the MFO catalysing the conversion to toluquinol from *m*-cresol being activated by agitation of the cultures.

Table 8. Toluquinol specific production in YEGB medium.

Time (h)	Shake (peak area/g dry weight) x 10^5	Static (peak area/g dry weight) x 10^5
24	-	277.8
48	5.6	-
72	8.5	3.1
96	3.1	30.9

HPLC detection is more sensitive, therefore the static concentrations can be detected, whereas the less sensitive paper chromatography/ spectrophotometric would not pick this up.

Citrinin was only detected at 96 hours in static cultures in this medium, and at 72 and 96 hours in the shake cultures. It was not detected at all in paper chromatography and spectrophotometric analysis. It is likely that this is due to the sensitivity of the HPLC method, and because in the spectrophotometric analysis the citrinin peaks tended to be obscured by larger peaks associated with other metabolites. A possible reason for the lack of citrinin accumulation in YEGB cultures is discussed later in Section 5.6.

5.5.3.2 ME medium results.

Differences were observed between the results for HPLC analysis, and spectro-photometric and paper chromatography analysis of culture filtrate extracts of *P. expansum* grown in ME medium. Toluquinol was only detected in 96 hour shake cultures and from 24 hours in the static cultures, whereas the paper/spec analysis showed toluquinol to be present in all shake cultures in this medium (Table 10). The 96 hour shake culture accumulated a broader array of metabolites, while the 24 hour static culture predominantly accumulated early pathway metabolites.

6-MSA was not detected routinely in this medium but there were sporadic appearances (i.e. not in every HPLC analysis of different culture runs) in static cultures over 48 hours old only. The appearance of this compound in late static cultures using HPLC corroborates with the specific patulin production data (Section 5.5.4) which shows the specific patulin levels in the culture medium (i.e. quantities derived on a dry weight basis) to be greater in static cultures than shake cultures. The nutrient poor medium would be likely to cause the organism to have lower precursor concentrations and therefore accumulation of these is less likely if metabolism was directed towards patulin production.

m-Hydroxybenzyl alcohol was detected in all cultures, but the shake culture peak areas were usually an order of magnitude higher than static cultures, indicating an increased level in these cultures. Rough calculations for specific production levels on

the basis of average culture dry weights and peak areas from a single HPLC run show the levels to be in the same magnitude for shake and static (Table 9). As mentioned in Section 5.5.3, the low static dry weights to gave rise to inflated static specific production values. An interesting observation in these specific results was that the shake cultures appeared to increase in their specific production of *m*-hydroxybenzyl alcohol whereas in the static cultures this activity declined.

Table 9. Specific production of *m*-hydroxybenzyl alcohol in ME medium.

Time (h)	**Shake (peak area/g dry weight) x 10^6**	**Static (peak area/g dry weight) x 10^6**
24	16.2	104
48	21.7	18.7
72	18.9	10
96	28.5	7.5

Citrinin was another compound that appeared at a completely different time to that seen in the paper/ spec analyses, again its appearance was only sporadic. HPLC analysis showed that citrinin appeared to be present in shake cultures at 72 and 96 hours, and not in static cultures. Paper chromatography/ spectrophotometric analysis showed citrinin to be present in static cultures after 24 hours. The regulation of citrinin metabolism is discussed in Section 5.6.

Table 10. Toluquinol specific production in ME medium.

Time (h)	**Shake (peak area/g dry weight) x 10^6**	**Static (peak area/g dry weight) x 10^6**
24	-	235
48	-	16.8
72	-	3.1
96	0.90	2.4

5.5.4 HPLC analysis of secondary metabolism in *P. expansum*: Quantitative analysis.

5.5.4.1 YEGB medium results.

Figure 45 shows the patulin levels that were measured using HPLC of culture filtrates of *P. expansum* grown in YEGB medium. Virtually no patulin was produced in the first 24 h in either shake or static cultures and only small amounts were produced in both at 48 h. The levels of patulin were increased significantly in the static 72 h cultures to an average concentration of 6.02 mg/L, resulting in the steep increase in the curve gradient at this time, whereas there was no average increase in the shake cultures. The 96 h static cultures maintained this high level of patulin concentration compared to the shake culture, which continued its slow increase in concentration to an average of 0.83 mg/L.

The day 1 static culture contained a substance (originally assumed to be patulin) which eluted 30-60 sec after the retention time expected for patulin. It is possible that this could be due to the 'yellow' compound that consistently appeared in paper chromatography at this time, when patulin was usually absent. This interfered with the patulin absorbance, inflating the 24 h static value, which should be low given that this culture would be experiencing an initial lag in growth at this time.

The specific production of patulin (i.e. mg patulin/g dry weight) by YEGB cultures of *P. expansum* is presented in Figure 46. In shake cultures this was very low and constant after 24 h because there was a significant increase in biomass in this organism under these conditions. The peak in the static results at 24 h should be ignored since this reflects the interference of precursors (mentioned above) with what should only be a negligible amount of patulin. In combination with the very low dry weight that was recorded (the average dry weight was 68 mg), an inflated false value resulted. A period of rapid growth between 24 and 72 h in the static cultures matched an increase in the specific patulin production, since there was a substantial increase in patulin concentration in the culture filtrate at this time. The increase in the patulin concentration at 72 h resulted in an increase in the specific production at this time,

since this was not mirrored by a large increase in biomass. The 96 h static specific production increased further, reflecting a slowing in the rate of biomass production.

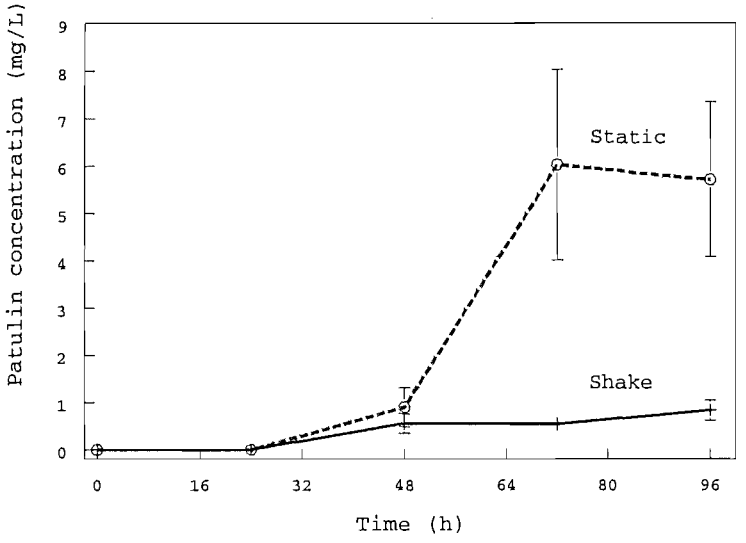


Figure 45. The concentration of patulin in culture filtrates of *P. expansum* grown in YEGB medium.

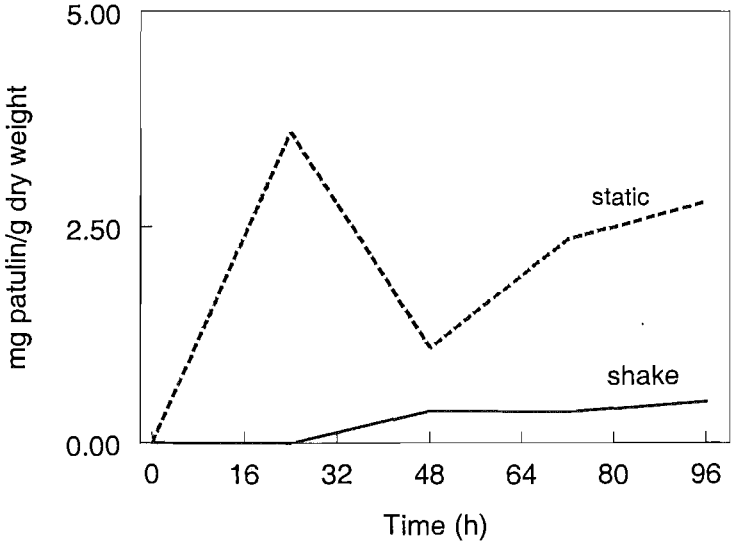


Figure 46. The specific production of patulin by *P. expansum* cultures grown in YEGB medium (on a dry weight basis).

5.5.4.2 ME medium results.

The production of patulin by *P. expansum* grown on ME medium is shown in Figure 47. These results follow a different pattern to those determined in the YEGB medium. At 24 h the shake cultures showed no patulin production whereas the static cultures were producing small amounts of a compound absorbing at the same wavelength as patulin. By 48 h the shake cultures were producing a small amount of patulin, slightly lower than the static culture concentrations. At 72 h both shake and static cultures showed an increase each of approximately three times that of the 48 h cultures. Figure 47 indicates that the standard errors overlapped for shake and static cultures from this time onwards. By 96 h the shake culture patulin concentration was approximately equal to the static culture since the static production was beginning to level out at this time, while the shake culture continued a steady increase in patulin levels.

Despite similar patulin concentrations being measured in the shake and static cultures, the picture is different when the biomass of the respective cultures is taken into account (Figure 48). Once again, there was an anomalous sharp peak in the specific production of patulin at 24 h in the static cultures, for the same reason given in Section 5.5.4.1, and should be zero. At 48 h the static culture specific activity was greater than the shake 48 h cultures. This increased further at 72 h, but decreased slightly at 96 h since there was only a small increase in culture filtrate patulin concentration coupled to an increase in dry weight at this time. The shake cultures remained at low specific production levels because of the greater dry weights throughout the culture period under these conditions.

The specific patulin production of the ME cultures was considerably greater than the YEGB for both shake and static cultures, despite the YEGB cultures accumulating greater levels of patulin in the culture medium. An average of 6.02 ± 2 mg/L for static day 3 cultures was the YEGB peak, whereas 3.2 mg/L ± 1.4 in shake 96 h cultures was the highest ME concentration recorded. Over double the levels of the YEGB cultures in the static cultures were calculated for the ME cultures in terms of specific production. Thus it can be concluded that the ME cultures were better at patulin production, and in both types of media aeration resulted in significant differences in

the specific patulin production. However the differences were more marked in the ME medium.

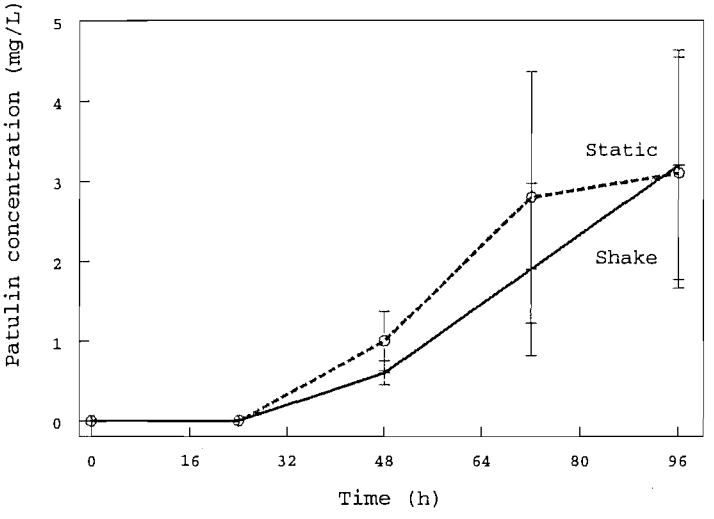


Figure 47. The concentration of patulin in culture filtrates of *P. expansum* grown in ME medium.

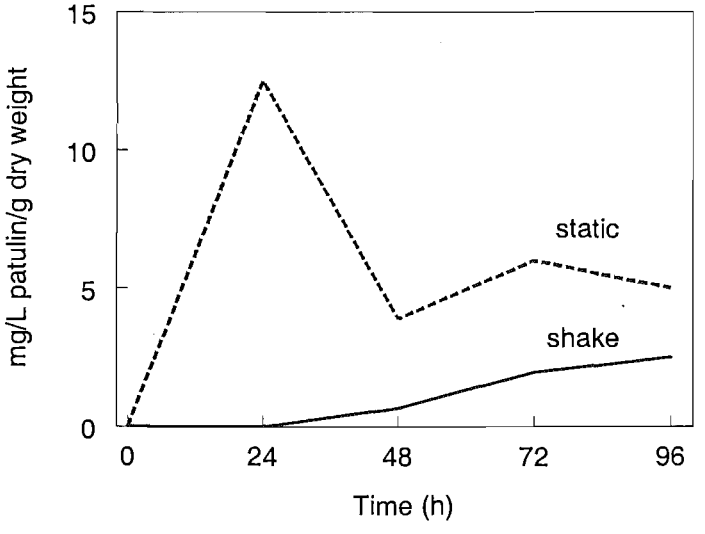


Figure 48. The specific production of patulin by *P. expansum* cultures grown in ME medium (on a dry weight basis).

5.6 SUMMARY

5.6.1 Patulin Biosynthesis.

Tables 11, 12, 13 and 14 summarise, on a day to day basis, the secondary metabolism events for *P. expansum* grown on ME and YE media. In the YEGB shake cultures the early portion of the patulin pathway was present in the day 1 cultures. 6-MSA and *m*-hydroxybenzyl alcohol were detected, but not toluquinol. The absence of toluquinol was consistent with the higher K_m for the *m*-cresol substrate for the MFO producing this (Priest and Light, 1989), compared to that which produces *m*-hydroxybenzyl alcohol, particularly under conditions when very little precursor would be present for the two enzymes to compete.

Only a small amount of patulin was detected in day 2 YE shake cultures, and there was a general decrease in the levels of the early pathway metabolites. Toluquinol was detected, indicating perhaps that activation of the MFO producing it by O_2 had occurred. The patulin biosynthetic pathway could not be considered important at this point in time in these cultures. Day 3 shake cultures gave a similar picture to day 2. The specific production levels for the monitored metabolites were decreased or the same as those for day 2, indicating that patulin biosynthesis was still not important metabolically in this organism. Toluquinol production appeared to remain significant, agitation still played a role in the activity of this MFO.

Day 4 YE shake cultures showed increased specific production levels compared to previous days, with the exception of toluquinol, which decreased. It is possible that there was more of a flux of precursors available to this pathway at this time, resulting in increased specific production levels. The decrease in the toluquinol could possibly be due to a change in the dissolved O_2 concentration in the medium at this time, and this is likely given the morphology of the fungus at this stage of growth in the type of medium. The fungus grew as a thick hyphal suspension, which showed decreased viscosity to previous days and presumably the O_2 transfer was decreased significantly. This is discussed further in Chapter 3. The change in DO conditions might also explain the increase in secondary metabolism in this organism. The fact that citrinin

was produced in some day 4 shake cultures as well as day 3, possibly corroborates this notion.

In general, the YEGB shake cultures results indicate that patulin production and pathway metabolite accumulation does occur but not to any great degree. The significance is discussed later in the Concluding Discussion (Chapter 8).

A feature of the day 1 YEGB static cultures was that there were high specific production levels due to the very low dry weights at this time, whereas only low levels of secondary compounds accumulated in the medium. Toluquinol was produced in reasonable levels in some cultures, which is perhaps unusual given the lack of agitation in these cultures. However, in the static cultures at this time surface growth would not be a significant hindrance to O_2 dissolving into the medium, so the DO levels in the medium were likely to be the highest for any time during the static culture period. A compound, possibly a patulin precursor or "yellow compound" was present at this time.

The specific production levels decreased on day 2, with the exception of patulin, which increased. Accumulation of early pathway metabolites tended to be strongest to this point, however beyond this time, flux through to patulin production began to increase. Day 3 static cultures represent a point at which patulin production became more dominant, with high levels accumulating by this time and an increase in its specific production. There was much less accumulation of precursors, indicating a general shift in metabolism towards patulin production.

There was an increase in specific production of pathway metabolites in day 4 YE static cultures, while the patulin concentration did not increase much due to a decrease in specific production. It appears that patulin production had stopped, resulting in accumulation of its precursors and this may indicate a saturation point for patulin production; possibly the patulin had fed back onto an enzyme late in the pathway to slow its accumulation.

The increase in patulin accumulation on day 3 in the YE static cultures was coincident with thickening of the mycelial mats growing on the surface of the cultures, as well as sporulation which was heavy by day 4. The specific production (Figure 46) is greater in the static cultures than the shake ones, more significantly at 72 and 96 hours. In order to make a more informed assessment of the degree of the feed into secondary metabolism, these results are discussed further in the Concluding Discussion (Chapter 8).

A small amount of the early pathway metabolite *m*-hydroxybenzyl alcohol was detected in day 1 shake cultures grown in ME medium, toluquinol was only picked up on paper chromatography. 6-MSA was not detected in any shake or static cultures grown in ME medium. Patulin was not detected at this time.

Day 2 ME shake cultures showed slightly increased evidence of secondary metabolism. The specific production of *m*-hydroxybenzyl alcohol was increased, along with an increase in the accumulated concentration. Toluquinol was present in some cultures but not at great levels and patulin was present only at small levels. Secondary metabolism would appear to be very slow and favouring only early pathway production at this point.

A reasonable increase in patulin accumulation in day 3 shake cultures indicated the increase in the pathway flux through to the end. There was not any great increase in specific production of *m*-hydroxybenzyl alcohol, indicating that the precursors would all be feeding through to patulin.

Day 4 ME shake cultures underwent a strong increase in the general level of secondary metabolism since there was an increase in *m*-hydroxybenzyl alcohol specific production, toluquinol was detected by HPLC and the patulin medium concentration continued to increase, although the specific activity did not increase greatly for this compound. Obviously secondary metabolism had increased its importance in this organism at this stage.

In terms of culture morphology, the day 4 ME shake cultures tended to be pigmented and there were usually a reasonable number of small mycelial pellets present. The formation of pellets would be in response to depletion of the nutrient poor medium. The pellets would almost certainly have a low O_2 internal environment, favouring secondary metabolism coupled to a growing exterior layer.

The ME day 1 static cultures showed secondary metabolism was occurring almost from the beginning of the culture. Because of the extremely low biomass present at this time, all the specific production levels were very high. Patulin was detected at this point in time as well by both HPLC and paper chromatography, indicating that secondary metabolism was fully established early in the static cultures. The presence of toluquinol could be explained in a similar manner to the static day 1 YE cultures, in that at this point in time the DO level may favour the activity of the MFO catalysing its production from *m*-cresol. The activation of secondary metabolism at this stage is a reflection of the extremely slow growth rate at this stage of the culture and also because factors such as ammonium repression would have been non-existent at this point in time given the nutritionally poor status of the medium. Ammonium concentration and other regulatory factors are discussed further in the Concluding Discussion (Chapter 8).

The specific production of *m*-hydroxybenzyl alcohol, toluquinol and patulin decreased on the second day of the static cultures, together with the actual medium concentrations not increasing. It may be that the increase in biomass meant that precursors were required by primary metabolism more than by secondary metabolism It may be that primary metabolism operates to feed secondary metabolism under these conditions and this became unbalanced upon growth.

Once again, the day 3 static cultures were responsible for a strong increase in patulin production. The precursor specific production levels were reduced since metabolism appeared to be directed to the end of the pathway and patulin production. Secondary metabolism was fully operational at this time.

Decreases in specific production were seen at day 4 in ME static cultures, and the accumulated levels of *m*-hydroxybenzyl alcohol and toluquinol did not increase, patulin production decreased also. This may be a result of the nutrients in the medium having been depleted and there being no input through the pathway. A feedback mechanism may also have been operating in which late pathway enzymes are inhibited by patulin to reduce production.

Lack of agitation and the low nutrient concentration of the medium both contributed to the ME static cultures favouring secondary metabolism from an early stage. The medium had a very low concentration of ammonium ions (see Chapter 6), which meant that the organism favoured polyketide biosynthesis if the metabolic rate was particularly slow. By comparison, the shake cultures would have had a higher rate of substrate utilisation by primary metabolism due to the presence of O_2. This is discussed more fully in the Concluding Discussion (Chapter 8).

In addition, as mentioned, in the introduction to this section, it would have been useful to determine the activities of enzymes responsible for polyketide biosynthesis. 6-MSA synthase (Grootwassink and Gaucher, 1980), 6-MSA decarboxylase (Light and Vogel, 1975), *m*-hydroxybenzyl alcohol dehydrogenase (Forrester and Gaucher, 1975), and isoepoxydon dehydrogenase (Sekiguchi and Gaucher, 1979) activities have been successfully detected and determined in *P. urticae*. Attempts to assay *m*-hydroxylbenzyl alcohol dehydrogenase were unsuccessful, since interference from another enzyme utilising endogenous substrates in the crude enzyme extracts occurred, which was identified in the assay controls and attempts at isozyme gels. Substrates were not available for the isoepoxydon dehydrogenase and 6-MSA synthase assays, the latter due to the prohibitive cost of radiolabelled malonyl CoA. Studies using probes for mRNA of these activities would also be very useful in determining *de novo* enzyme synthesis as a result of secondary metabolic gene derepression. Further studies on the identity of the 'yellow compounds' produced by reaction with MBTH on paper chromatograms could be facilitated by the generation of mutants, however this was beyond the scope or the time restraints of this project.

5.6.2 Citrinin Biosynthesis.

The results presented here showed differences between the three methods used in the qualitative detection of citrinin. Paper chromatography indicated that there was no citrinin produced in YE culture filtrates, and it was not detected by spectrophotometric analysis in either medium. However, some citrinin was found in 96h static cultures, and 72 and 96h shake YE cultures using reversed-phase HPLC analysis. The HPLC method is the most sensitive of the three and was better able to separate citrinin from other compounds compared to spectrophotometric analysis, in which the citrinin peak was frequently be obscured by peaks from larger concentrations of other metabolites in the extracts.

HPLC analysis of the ME cultures showed citrinin was present only in 72 and 96h shake cultures. Paper chromatography indicated it was present in the static cultures at these times as well.

The later appearance of citrinin in cultures of *P. expansum* indicates that it is under a different regulatory control from patulin biosynthesis. None of the postulated citrinin intermediates were monitored in this work (e.g. sclerotinin A and dihydrocitrinone), so complete conclusions cannot be drawn about the pathway.

In general terms, citrinin production appears to be favoured more in shake cultures of both media than in static. This is contrary to the trends noted in patulin biosynthesis, which is favoured more by the static cultures. Both are derived from acetyl CoA precursors, and the differences could be accounted for by the relative affinity for this substrate by the polyketide synthases.

In shake cultures, the production of the citrinin PKS could be stimulated. This may be in response to a signal from primary metabolism and could include citrate, or the cofactor involved in citrinin synthesis, methyl methionine. If the citrinin PKS is more active at a given time than the patulin PKS, the former will preferentially utilise the acetyl CoA precursor, along with fatty acid biosynthesis.

It is probable that citrinin production is under a separate form of regulation to patulin biosynthesis. Griseofulvin production by *P. urticae* is under catabolite repression, compared to ammonium repression of patulin synthesis (M. Gaucher, *pers.comm.*). Catabolite repression was considered as a regulatory mechanism for citrinin in *P. expansum*, however the production of citrinin in shake cultures, and in YE medium, conditions which would tend to favour excess catabolite production, negate this proposal. Regulation by ammonium at different concentrations to patulin repression was considered, but disregarded because citrinin production tended to occur in shake cultures whereas patulin occurred in static, instead of later in the statics as one might expect if the ammonium ion repression threshold was lower for citrinin production.

The feed of substrate to the citrinin biosynthetic pathway would also affect production. Given a situation in which the citrinin PKS is activated in some way by agitation, it is possible that provided this enzyme was activated to give an affinity for the substrate similar to fatty acid synthase, and greater than patulin PKS, the levels of substrate available to these enzymes would dictate the level of citrinin synthesis possible. Along similar lines, the level of methyl methionine which may have been limiting could be increased by agitation and therefore allow the production of citrinin to proceed.

Metal ions provide another potential means for regulation of citrinin synthesis. The catalytic ability of citrinin biosynthetic enzymes could be enhanced or inhibited by conformational changes brought about by metal ion binding or associations. If this was the case, citrinin synthesis would occur either more or less readily in the ME medium than the YE medium because the former is not supplemented with metal ions. Depletion of an inhibitory metal ion may explain why the day 3 and 4 shake cultures produced citrinin. However, citrinin was still produced in the supplemented YE cultures, seemingly contradicting this.

A final possibility is that the production of citrinin and patulin occur at different intracellular locations and therefore the relative flux to these and the pool of available precursor would dictate how much production of each of these polyketides is possible.

It is difficult to draw conclusions about the regulation of citrinin biosynthesis given that no quantitative analyses were performed in this work. There are a limited amount of studies that have examined citrinin biosynthesis. Betina *et al.* (1973) showed that citrinin production in *P. notatum* Westling S-52 was biphasic, occurring in both tropo- and idiophase. The production of citrinin in *P. notatum* was regulated by the utilisation of acetyl CoA by primary metabolism, and the levels of citrinin and total lipid peaked at opposite time to each other. In the experiments with *P. expansum*, citrinin production appeared to coincide with lipid accumulation in the shake cultures, particularly in the ME medium. This is contradictory to conclusions that primary metabolism usage of acetate affects citrinin synthesis.

Blanc *et al.* (1985) showed that citrinin production in *Monascus ruber* was favoured by a low C/N ratio in the culture medium. This would imply that the high levels of glucose in the YE medium would be less favourable for citrinin production than in the ME medium.

Table 11. Summary YEGB medium shake cultures; intermediates of patulin biosynthesis

Metabolite	Shake Day 1	Shake Day 2	Shake Day 3	Shake Day 4
6-MSA	Highest specific level of the shake cultures, highest accumulated levels of the shake cultures	Not detected (by HPLC & paper chromatography)	Lowest specific level of shake cultures (except day 2 when not detected), although not much lower than shake 1 levels	Specific production and accumulated concentration increased from day 3
m-Hydroxybenzyl alcohol	The highest of the shake cultures in terms of specific production; highest concentration in medium	Levels decreased from shake 1, not detected on paper	Lowest specific production of shake cultures, but increased accumulated concentration to previous days	slight increase in specific production, not detected in paper
Toluquinol	Not detected (HPLC & paper chromatography)	Moderate specific production, reasonable levels accumulated (Paper detected)	Increased accumulated levels over previous 2 days, increase in specific production, paper detected	Decreased specific production and accumulated concentration, detected by paper
Patulin	Not detected (HPLC & paper chromatography), but "yellow" compound produced	small amount detected by HPLC, detected on paper, low specific production	Small amount detected by HPLC, similar level and specific production to day 2	slight increase in specific production and concentration over day 3

Table 12. Summary YEGB medium static cultures; intermediates of patulin biosynthesis

Metabolite	Static Day 1	Static Day 2	Static Day 3	Static Day 4
6-MSA	Low levels, high specific production, not detected by paper	Order of magnitude lower than on day 1 for specific production, increased accumulation in medium	Decreased specific production but increased accumulation	significant increase in specific production, greater accumulated concentration
m-Hydroxybenzyl alcohol	Specific production highest of the static cultures, but lowest medium concentration	Specific production order of magnitude lower than day 1, increased accumulation in medium	Decreased specific production but increased accumulation, detected on paper	only detected on paper
Toluquinol	Huge specific production, high levels accumulated, not detected by paper	Not detected HPLC or paper	Decreased specific production and decreased accumulation	high specific production (but still an order or magnitude lower than static 1), accumulated level higher than other days
Patulin	"yellow" compound, something at that λ on HPLC	Increased specific production and accumulated levels	Leap in accumulated concentration, increased specific production	accumulated concentration approximately the same as day 3, slight increase in specific production (drop in average biomass)

Table 13. Summary ME medium shake cultures; intermediates of patulin biosynthesis.

Metabolite	**Shake Day 1**	**Shake Day 2**	**Shake Day 3**	**Shake Day 4**
6-MSA	Not detected by HPLC or paper	Not detected	Not detected	Not detected
m-Hydroxybenzyl alcohol	Lowest specific production, not detected by paper	Increase in specific production and accumulated concentration, detected on paper.	Specific production not changed but increased accumulated amount, detected by paper	Specific production significantly increased and big increase in accumulated concentration, detected in paper
Toluquinol	Not detected by HPLC, possible detected by paper	Not detected by HPLC but detected by paper	Not detected by HPLC but detected by paper	Very low specific production and accumulated concentration compared to statics, detected by paper
Patulin	Not detected	Small amount detected, very low specific production, detected by paper	Reasonable increase in accumulated concentration, small increase in specific production, detected by paper	Continued increase in accumulated concentration, at a similar rate to days 2-3, only slight increase in specific production, detected by paper

Table 14. Summary ME medium static cultures; intermediates of patulin biosynthesis.

Metabolite	**Static Day 1**	**Static Day 2**	**Static Day 3**	**Static Day 4**
6-MSA	Not detected	Not detected	Not detected	Not detected
m-Hydroxybenzyl alcohol	Very high specific production, moderate accumulated levels, detected by paper	Big drop in specific production (an order of magnitude), but still reasonable level, increase in accumulated level, detected by paper	Specific production decreased, increase in accumulated level	Decreased specific production, maintained accumulated level
Toluquinol	Very high specific production, highest accumulated level, not detected on paper	Big decrease in specific production (order of magnitude), decrease in medium concentration from static 1, not detected by paper	Decrease in specific production (order of magnitude) to low level, decrease in accumulated level from static 2, not detected in paper	Slight decrease in specific production (low), decrease in accumulated level from day 3, not detected in paper
Patulin	Reasonable but small amount detected, big specific production, detected by paper (yellow spot too)	Big decrease in specific production, still at a reasonable level, medium levels approximately the same	Reasonable increase in accumulated levels, increased specific production	Small increase in accumulated levels, specific production decreased to a small degree

CHAPTER 6

THE EFFECT OF AGITATION ON EXTRACELLULAR ACTIVITIES OF *PENICILLIUM EXPANSUM.*

6.1 INTRODUCTION

The composition and properties of the culture medium of filamentous fungi can reflect the metabolic state of the organism since fungi digest their growth substrate extracellularly before absorption into the mycelium. Medium pH, ammonium ion concentration and polygalacturonase activity were not only indicators of metabolic activities of *Penicillium expansum*, but also parameters monitored in support of investigations undertaken in other chapters. For the culture conditions employed in this work please refer to Chapter 3.

The primary objective for work undertaken in this Chapter was to quantify ammonium ion concentration, extracellular polygalacturonase activity and and medium pH, in order to relate to primary and secondary metabolism results so that a clearer picture of the effects of agitation on *P. expansum* can be established.

6.2 AMMONIUM IONS

6.2.1 Introduction

The ammonium concentration was monitored principally to indicate whether or not repression of secondary metabolism by nitrogen was occurring. Nitrogen repression (reviewed in Chapter 2) has been shown to play a major role in the regulation of patulin biosynthesis in *Penicillium urticae* (Grootwassink and Gaucher, 1980;

Gaucher *et al.*, 1981; Deo and Gaucher, 1985; Rollins and Gaucher, 1994), despite the absence of N in this polyketide.

Rollins and Gaucher (1994) added milli-molar concentrations of ammonium chloride to Yeast Extract Glucose Buffer (YEGB) medium (the same as that used in these studies) to demonstrate the repressive effects of NH_4^+ on pathway enzymes and the formation of patulin pathway metabolites, as well as intracellular proteinase activity in *P. urticae*. Chemostat cultures of this fungus showed that reduction of the external ammonium concentration from 30 mM to 4 mM was required to initiate the induction/derepression process and the first pathway enzyme was derepressed at 2.5 mM. Addition of NH_4Cl prior to idiophase resulted in delaying the appearance of the patulin biosynthetic pathway, and addition during idiophase caused the cessation of enzyme accumulation. The regulation of intracellular proteinase activity was also observed with NH_4^+, although this was less sensitive to regulation than the polyketide genes.

The concentrations of ammonium ions in the two media used in this study were measured to indicate whether these were likely to be at a level that could potentially repress secondary metabolism in *P. expansum*. If the levels were sufficiently low, then it could be assumed that the effects on secondary metabolism observed with agitation were more likely to be due to that effect alone. If the levels of ammonium were approximately those which might lead to repression of secondary metabolism, assumptions could then be made regarding the aeration conditions which could lead to derepression occurring, since it would be likely that ammonium would be utilised at different rates. The utilisation of ammonium would also serve to reflect the growth rates and requirements of *P. expansum*.

Ammonium can be generated from amino acids by mechanisms such as oxidative deamination following transamination. 2-Oxoglutarate is transaminated to glutamate, which is then deaminated by glutamate dehydrogenase to give α-ketoglutarate and NH_4^+. This reaction is normally mediated by the cofactor, pyridoxal phosphate. By comparison, the direct deamination by enzymes such as serine and threonine

dehydratases is not mediated by this cofactor; rather dehydration followed by deamination occurs to yield pyruvate from serine and α-ketobutyrate from threonine.

Table 15 shows the contribution of peptone and yeast extract to total and amino N levels in ME and YEGB media respectively. Amino nitrogen refers to the levels of amino groups in the media, which is a direct reflection of the concentrations of protein and amino acids. Total N refers to all nitrogen-containing compounds in the media.

Table 15. Total and amino nitrogen concentrations of media components.

Parameter	Peptone (ME)	Yeast Extract (YEGB)
Concentration in medium (g/L)	3	5
Total N in dry powder (%)	15.5	10.9
Total N in final medium (%)	0.05	0.01
Amino N in dry powder (%)	3.1	6
Amino N in final medium (%)	0.01	0.03
Amino N in final medium (mM)	5.2	16.7

(Information supplied by Difco)

There are significantly greater levels of amino N in the YEGB medium than in the ME medium. This information is considered with the ammonium quantification results.

6.2.2 Phenol-hypochlorite reaction for the determination of ammonium ions.

This assay was based on that of Weatherburn (1967) as used by Rollins and Gaucher (1994). Ammonium standard (20 µL) or appropriately diluted sample was added to 5 mL reagent A and mixed thoroughly. 5 mL of reagent B was added and mixed thoroughly and incubated for 20 minutes at 37 °C. The absorbance at 625 nm was recorded and compared to standards. Standards contained dilutions of a 1 mg/mL solution of ammonium sulfate or 0.9 mg/mL ammonium chloride to give 0.5 - 6 µg NH_4^+. Reagent A was a 1 % w/v solution of phenol in water containing 50 mg/L sodium nitroprusside. Reagent B was prepared by dissolving 2.5 g NaOH in a

solution consisting of 4.2 mL (undiluted) sodium hypochlorite made up to 500 mL with water.

6.2.3 Results and Discussion.

Figures 49 and 50 show the medium ammonium ion concentrations for ME and YEGB media respectively. The Malt Extract medium NH_4^+ concentration was very low and consequently the standard errors appear quite large in these results. The shake cultures underwent a decrease in NH_4^+ concentration up to 48 hours, followed by an increase. It may be that at this point is a lower threshold level that the organism strives to maintain by deamination of medium components or through discharge of intracellular material. This level is possibly the lowest the ammonium concentration can reach within the limits of the assay. The lowest NH_4^+ concentration used by Weatherburn *et al.* (1967) 0.5 μg, or 1.4 mM. The results for the ME medium in this work were below this.

The ME static culture media increased in ammonium concentration at 24 h, indicating that ammonium was being released from the amino nitrogen compounds in the medium for utilisation by the organism. This was followed by a gradual decrease in concentration to the same level as the shake cultures at 96 h. Since the levels of this nutrient appear the same at 96 h, it could be concluded that it does not drive growth since the decrease in concentration does not reflect increases in biomass accumulation.

The medium ammonium concentrations of the YEGB *P. expansum* cultures were higher than the ME medium ones, yet the concentrations were still lower than 1 mM. The shake cultures utilised the ammonium concentration rapidly to 48 h, followed by a very slight increase to 96 h. Once again, in the static 24 h cultures there was an increase in ammonium concentration compared to the original medium. This was followed by very rapid depletion in levels up to 72 h and levelling off at 96 h, presumably because this nutrient was nearly depleted.

Neither of the culture media used here contained high levels of ammonium ions. The levels recorded were significantly lower than the levels at which derepression of

patulin biosynthesis occurred in *P. urticae* (Rollins and Gaucher, 1994). Therefore any effects of agitation on secondary metabolism in *P. expansum* are not likely to be a result of nitrogen repression and nitrogen utilisation by the fungus. Therefore it is more likely that the secondary metabolism could occur from the outset of growth.

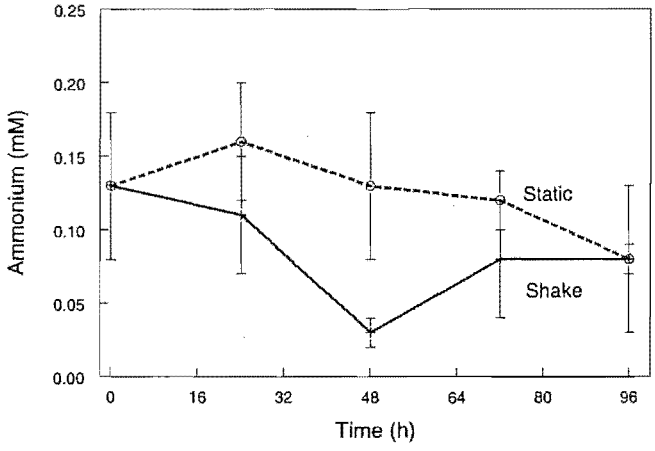


Figure 49. Ammonium concentration in ME medium.

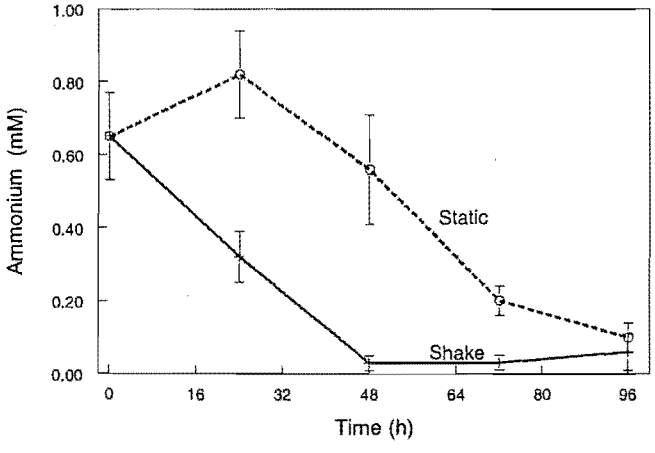


Figure 50. Ammonium concentration in YE medium.

6.3 MEDIUM pH

6.3.1 Introduction

Medium pH was also monitored in these experiments since filamentous fungi prefer acidic environments for growth and tend to acidify the medium in which they are growing. The pH optima of many extracellular fungal enzymes are in the acidic range. One objective of monitoring the pH of the culture medium was to determine whether the culture medium pH could be responsible for the destabilisation of patulin. Patulin is a lactone and is therefore prone to hydrolysis at a pH greater than 6. Measurements of media pH were included to establish whether some unidentified compounds present in ethyl acetate extracts could be related to pH mediated degradation of patulin.

Pimentel *et al.* (1996) indicated that K^+ concentration and pH play an important role in citrinin production in *Penicillium citrinum* as a consequence of studies to produce lipase free of citrinin, however they did not offer an explanation or possible mechanism for this effect. The K^+ and pH effects were observed when glycerol was replaced as the carbon source by olive oil, soybean oil and corn oil. Citrinin was produced when the pH was raised to 6.5 from 4.5 and the K^+ concentration to 50 mM from 30 mM. These effects may not be observed in *P. expansum* using the ME and YEGB media, however it is important to consider the possibility that some kind of pH regulation may exist.

The pH conditions of the culture medium may reflect an intracellular requirement for a particular reduction-oxidation state. A sharp increase in medium pH late in culture growth is an indication of autolysis occurring and cellular contents (normally at a more neutral pH) being released from the mycelium. The rate at which the medium pH changes would provide a reasonable indication of the relative growth rates and phases of the fungus.

6.3.2 Medium pH measurement method.

The measurement of medium pH was performed with a glass electrode and pH meter at 20 °C.

6.3.3 Results and Discussion

As its title suggests the Yeast Extract Glucose Buffer medium was buffered and there was not a great degree of variation in the pH level during the growth of the culture (Figure 52). The shake cultures decreased in pH from 6.5 initially, to 5 after 48 h, then increasing only slightly after that. The static cultures lagged 24 h before exhibiting a decrease in pH to 4.8 after 72 h.

The decrease in YE pH levels appears to follow the growth kinetics of these cultures. The 24-hour lag in static cultures occurred for both dry weight and the medium pH change. The shake and static cultures had reached similar dry weights by 72 hours and this is reflected in similar pH levels under both conditions.

The pH exceeded 6 only in the initial medium and the 24 h static cultures. Thus it may be possible that any patulin produced in the 24 h static culture could be destabilised at this pH. This is discussed further in Chapter 5.5.1.1.

The ME medium (Figure 51) was a lower pH initially (5.9) than the YE medium. This medium was unbuffered and therefore the decreases in pH were more substantial than those observed in the YEGB medium. The shake culture pH decreased to 3 after 72 hours, with a very slight increase occurring after 96 hours. The static culture did not show the same lag as the YEGB static culture; instead, the fungus acidified the medium from the outset. The static culture medium pH underwent a gradual decrease to 3.4.

The ME medium pH decreases resembled the dry weight increases. The static culture decrease in pH did not reflect the 24 hour lag in growth at the start of the culture. Similarly the final pH of both the shake and static cultures did not reflect the

substantial difference in dry weight observed between these cultures. The fact that the pH did not change in the shake culture after 48 hours suggested that the medium had been acidified to a threshold level. It is likely that this threshold represents one extreme of the optimal range tolerated by this fungus. The ME medium pH never exceeded 6 and therefore it is unlikely to have an effect on the stability of patulin.

The pH of both types of media did not show any substantial increases in pH that would suggest that autolysis had occurred. It was unlikely that autolysis would have occurred during the relatively short-term 4 day culture period.

Acidification of the culture medium is the result of extracellular acid production. Some of the proposed intermediates of patulin biosynthesis would be expected to contribute, including 6-MSA, *m*-hydroxybenzoic acid and gentisic acid. Other sources include galacturonic acid released by polygalacturonase and TCA cycle intermediates such as oxaloacetate, citrate, malate, and succinate, for example.

Another potential contributor could be the redox environment of the medium through aeration. This would not necessarily contribute to the pH of extracts that had been stored prior to measurement, but could affect the oxidation state of metabolites in the medium. Acids are the most oxidised form of alcohols and aldehydes, and a change in oxidation state of the latter two and other compounds could result from interactions with oxidising agents. These could take the form of radicals generated in hyperoxidant states, or during periods of intense respiration. The release of protons during periods of rapid respiration is likely, and this would be reflected in the rapid acidification of the medium during growth.

It is also a possibility that culture medium pH reflects a reducing/oxidising state intracellularly. Since acids are more oxidised than alcohols and aldehydes, it follows that greater levels of one of these forms of this type of compound might be an indicator of the cell's internal redox environment. For example, hyperoxidant states caused by the production of excess reactive O species which are unable to be neutralised by the cell, or by entry of excess O_2 into the cell and therefore its metabolism, could contribute to processes such as protein carbonylisation through

oxidation (Hansberg and Aguirre, 1990). This could have been detected by measuring levels of redox/ O_2 scavenger molecules such as glutathione (oxidised and reduced forms – GSSG and GSH), as well as the activity of enzymes such as glutathione oxidase.

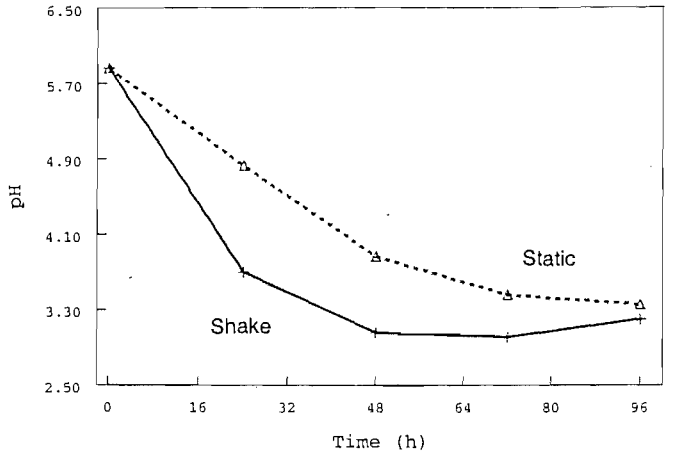


Figure 51. ME medium pH for *P. expansum* cultures.

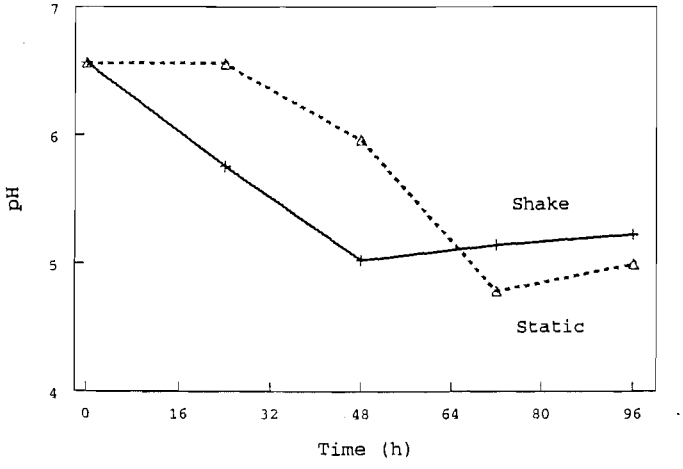


Figure 52. YEGB medium pH for *P. expansum* cultures.

6.4 POLYGALACTURONASE ACTIVITY

6.4.1 Introduction

Filamentous fungi produce a wide range of extracellular enzymes which digest the more complex compounds present in the growth substrate prior to uptake into the mycelium. Included amongst these are enzymes for proteolytic digestion and for the degradation of complex polysaccharides. *Penicillium expansum* is typically isolated from soft rots of fruit, in particular from apples, and therefore pectinolytic enzymes are important to the ability of the organism to access the available nutrients.

Pectin is hydrolysed by a group of enzymes known as pectinases by a number of different mechanisms, including those that attack methyl-esterified pectin (pectinic acid, for example pectinesterase), and those that act on de-esterified pectin (pectic acids). Within these classes there are hydrolases and lyases that cleave the polysaccharide randomly (endolytic) or from the ends of the chain (exolytic) (Acuna-Arguelles *et al.*, 1995). According to (Jansen and MacDonnell (1945), the ability of polygalacturonase to hydrolyse pectin was enhanced by prior de-esterification. Polygalacturonase production in *Rhizoctonia solani* was constitutive, and not affected by addition of validamycin A, the antibiotic used to control rice sheath blight, to cultures grown on cellulose as the carbon source (Robson *et al.*, 1989).

Polygalacturonase and pectinesterase are two such enzymes. Woodhead and Walker (1975) had shown previously that the extracellular polygalacturonase activity of *P. expansum* could be affected by aeration of liquid cultures. Polygalacturonase activity was greater in agitated cultures than for static ones, and was the principal pectinolytic activity, with very low pectinesterase activity being detected. *P. expansum* produces a soft, white rot of apples (Cole and Wood, 1961a). Cole and Wood (1961b) detected high pectinesterase activities in apple rots from the same organism. Measurement of this activity was included in these studies to indicate how the requirement for the fungus to access its surrounding medium was affected by aeration. It was possible that different results could be observed given the significant differences in the nutritional composition of the two media used.

Guevara *et al.* (1997) showed that there were multiple forms of pectic lyases and polygalacturonase produced by *Fusarium oxysporium* f.sp. *radicis lycopersici.* There were three pectic lyase activities (endolytic), four polygalacturonases and one pectinesterase (both types exolytic). Each differed in pI, the lyases requiring Ca^{2+}, whereas the polygalacturonases were inhibited by this ion. Both these types of enzyme were induced by galacturonic acid and were subject to catabolite repression. Using sequence analysis Ho *et al.* (1995) showed that a fragment of the *A. nidulans* pectate lyase *pelA* gene promoter could bind to the N terminal portion of CREA, a protein involved in carbon catabolite repression and glutathione-S-transferase, suggesting that CREA may contribute to the regulation of *pelA* gene expression.

The production of multiple forms of polysaccharide-degrading enzymes by a fungus would confer a greater adaptability to changing environmental conditions, or fulfil different tasks in the degradation process. Two pectinesterases were characterised from *Phytophthora infestans*, one of which may have contained two proteins, possibly forming a pectinesterase complex (Forster and Rasched, 1985). Whether or not *A. niger* was cultured on solid state or submerged media can affect the pectinase isozyme properties as well their production by different strains (Minjares-Carranco *et al.*, 1997). Use of different carbon sources in both submerged and solid state fermentations showed that regulatory phenomena of *A. niger* pectinase production were different in the two types of fermentation (Solis-Pereira et al., 1993).

6.4.2 Polygalacturonase assay method.

The polygalacturonase assay of Tagawa and Kaji (1988) was used, and consisted of an enzyme reaction followed by a subsequent chromogenic reaction with the reducing groups generated.

The reaction mixture included 2 mL 0.8-1% acid soluble pectin in pH 2.5 citrate phosphate buffer (0.1 M), 0.5 mL 0.1 M citrate-phosphate buffer pH 2.5 and 0.5 mL enzyme. The samples were mixed and incubated for 30 minutes at 30 °C, then stopped by the addition of 0.1 mL 1 M Na_2CO_3.

Acid soluble pectin was prepared as per McCready and Seegmiller (1954).

Sample (1 mL) was mixed with 1 mL copper reagent and heated in a boiling water bath for 15 minutes, then allowed to cool. 1 mL arsenomolybdate reagent was added to each sample and the absorbance was read at 500 nm.

Copper solution was prepared by mixing 4 volumes of Copper solution I with 1 volume of Copper solution II prior to use. Copper solution I consisted of 12g potassium sodium tartrate (Rochelle salt), 24g anhydrous Na_2CO_3, 16g $NaHCO_3$, and 144g Na_2SO_4 made up to 800 mL with water. Copper II solution was made from 4g $CuSO_4$ and 36g Na_2SO_4 made up to 200 mL with H_2O.

The arseno-molybdate colour reagent was made by adding 25g ammonium molybdate to 450 mL H_2O, followed by 21 mL concentrated H_2SO_4 with mixing. A solution of 3g Na_2HAsO_4 in 25 mL H_2O was mixed with the ammonium molybdate solution and incubated at 37 °C for 24-48 hours (or 55 °C for 25 minutes with stirring to prevent loss of chromogen).

One Unit is defined as μmoles galacturonic acid released per minute at 30 °C.

6.4.3 Results and Discussion

The polygalacturonase activity results were compared on a dry weight basis, and are shown in Figures 53 and 54. There was an initial peak of activity at 24 hours for both shake and static cultures, grown on both types of media. In both media the static 24h activity was significantly greater than the shake activities. Although the shake cultures had greater measured activities, the slower growth rate resulting from the lack of agitation meant that the activities in the static cultures were greater when related back to the biomass/dry weight.

The measured total activities in the filtrates of shake and static cultures did not differ greatly, since the errors overlapped when plotted with the results.

The greater activity of the ME static cultures could possibly be explained by the need for the organism to extract more carbon source from the medium due to the lower growth rate than the shake cultures, due to the slower feed of precursors through primary metabolism. Similarly, since the shake cultures would have a steady flow of precursors due to the presence of a terminal O_2 acceptor, there is a lesser requirement to access carbon from the medium through polygalacturonase activity.

The YEGB medium results were higher than those obtained in the ME medium. The YEGB medium contained more nutrients and was more complex than the ME medium. This may have required additional ability to break down the medium substrate.

This leads to three potential regulatory mechanisms for polygalacturonase. The first is carbon catabolite repression of polygalacturonase activity, which could be occurring in the shake cultures (particularly in the YEGB medium) through the increased presence of catabolites due to increased metabolic activity. The repression of polygalacturonase activity in shake cultures would arise since there would be a constant flow of metabolites through the favourable aeration conditions, and therefore less need for the organism to continue this feed. Guevara *et al.* (1997) showed catabolite repression of polygalacturonase activity in another filamentous fungus, *F. oxysporium.*

The second potential regulatory mechanism is the effect of metal ions to enhance or inhibit polygalacturonase activity. This could explain the better activities found in the YEGB medium, which contained trace metals, compared to the unsupplemented ME medium. Metal ion effects on polygalacturonase activity have been demonstrated previously by Guevara *et al.* (1997), who showed the inhibition of polygalacturonase activity in *F. oxysporium* by Ca^{2+}.

Thirdly, the possibility also exists that the YEGB medium contained an inducer for extracellular polygalacturonase, resulting in enhanced catalytic activity compared to the ME medium.

Another mechanism, which may exist but was not determined in this work, is degradation of the polygalacturonase enzyme by extracellular proteases. Intracellular protease termination of patulin biosynthesis has already been discussed in Chapter 5.1.4.3. It would not be inconceivable that a similar mechanism would be operating here, particularly since the extracellular proteases would be produced by the organism to digest proteins in the growth substrate. Polygalacturonase could be degraded by enzymes released concurrently or later for the specific purpose of assimilating proteins from the medium.

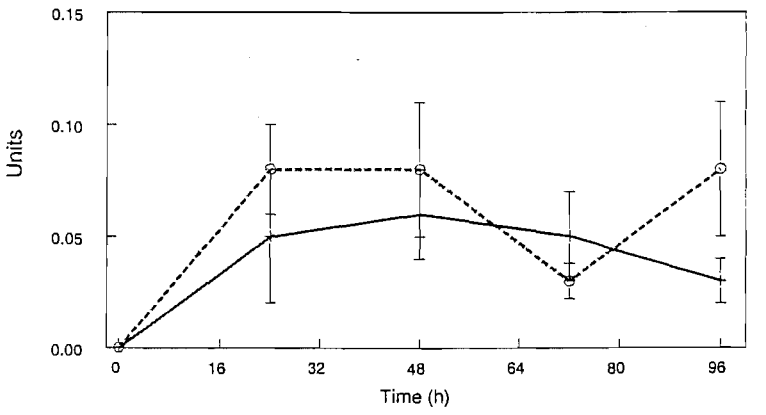


Figure 53. Polygalacturonase activity of ME cultures of *P. expansum*.

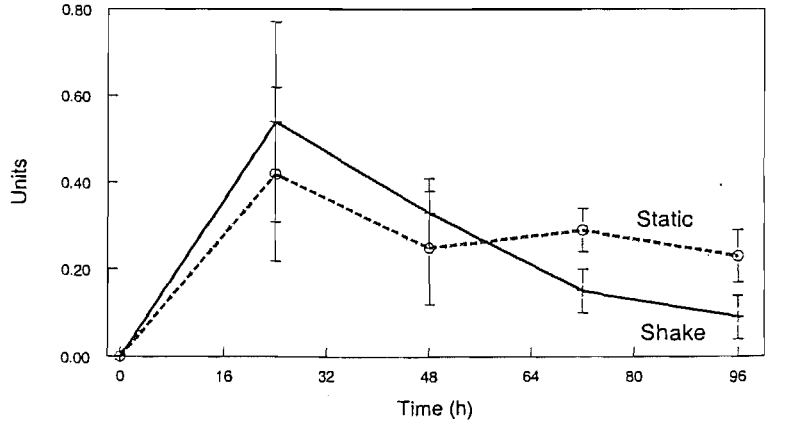


Figure 54. Polygalacturonase activity of YE cultures of *P. expansum*.

CHAPTER 7

AGITATION AND LIPID ACCUMULATION IN LIQUID CULTURES OF *PENICILLIUM EXPANSUM.*

7.1 INTRODUCTION

The biosynthesis of fatty acids involves the condensation of malonyl CoA with acetyl CoA by the multienzyme complex, fatty acid synthetase (FAS), in a process similar to the biosynthesis of polyketides through a series of orderly and progressive reactions. Synthesis of fatty acids occurs in the cytosol, whereas the degradation of fatty acids occurs in the mitochondrial matrix.

Intermediates in fatty acid synthesis are linked to acyl carrier protein (ACP). Lynen (1961) reviewed the FAS reaction and it is summarised in the following paragraphs. The nucleophilic methylene group of malonyl CoA adds to the electrophilic carbonyl carbon of the acetyl CoA thioester to produce a 5C intermediate, and is possibly activated further by an electrophilic centre of the enzyme. The intermediate is converted to the ketoacyl derivative by the elimination of the CO_2 and SH. NADPH is used as the cofactor in the reductive biosynthesis of fatty acids. Substitution of this with NADH results in a decrease in the rate of reaction by 75 %.

Acetyl CoA is an absolute requirement for the incorporation into the final product by the fatty acid synthetase. It acts as a "primer" for fatty acid synthesis onto which the malonyl CoA units are added. Acetyl CoA can be replaced by saturated acyl CoA molecules such as propionyl, butyryl, and hexanoyl CoAs, but not α,β-unsaturated, β-hydroxyl, and β-ketoacyl CoA derivatives.

The FAS enzyme contains sulfhydryl groups since the activity was stimulated by the addition of thiols such as cysteine and glutathione. The inability of workers to demonstrate free intermediates indicated that the mechanism involves a transfer of the acyl portion of the CoA esters to the sulfhydryl groups on the enzyme itself. The condensation product is a β-ketoacyl-enzyme rather than the β-ketoacyl CoA. The thermodynamic advantage that is gained by the decarboxylation of malonyl CoA would be lost through the thiolytic cleavage of acetoacetyl-CoA catalysed by the enzyme thiolase. Experiments using radiolabelling and mild alkaline hydrolysis released the acetoacetyl intermediate from the enzyme. The elongated chain is transferred back to CoA at some stage during the process.

The dehydration of the β-hydroxy-derivative is stereospecific. Studies using *S*-β-hydroxybutyryl *N*-acetyl-cysteamine as a substrate and following the characteristic absorption of dehydroacyl thioesters at 263 nm showed that only the α,β-unsaturated D-β-hydroxybutyryl thioester was dehydrated. Reduction with NADPH was shown using *S*-crotyl-*N*-acetyl-cysteamine.

The synthesis of fatty acids differs from the reversal of the fatty acid degradative cycle. This is important in keeping the synthetic process separated from the degradative pathway. Elongation of fatty acids is terminated at palmitate (C_{16}), subsequent chain elongation and desaturation is catalysed by other enzyme systems (Stryer, 1988). The direct elongation and desaturation of PUFAs in *P. chrysogenum* was demonstrated with substrates radiolabelled with ^{14}C (Bennett and Quackenbush, 1969).

Slabas *et al.* (1994) reviewed the pivotal reactions in fatty acid biosynthesis. The first was at acetyl CoA carboxylase (ACC), the enzyme catalysing the formation of malonyl CoA from acetyl CoA. This represents the first committed step in fatty acid synthesis. This enzyme is subject to strong control by phosphorylation/ dephosphorylation in animal systems, which in turn is affected by the nutritional status of the animal. It is also regulated by citrate and long chain acyl CoA. Activity of this enzyme in rape seed preceded the onset of lipid deposition and once the full complement of storage lipids had been obtained the enzyme activity declined rapidly.

The kinetics of induction were similar to FAS but the activity of that enzyme did not decay once a full complement of storage lipids had been obtained. ACC requires the presence of biotin in order to catalyse the reaction:

$$\text{Enzyme-biotin} + Mg^{2+} + \text{ATP} + HCO_3^- \leftrightarrow \text{enzyme-biotin-}CO_2^- + Mg^{2+} + \text{ADP} + P_i$$

$$\text{Enzyme-biotin-}CO_2^- + \text{acetyl CoA} \leftrightarrow \text{malonyl CoA} + \text{enzyme-biotin}$$

Several lines of evidence suggest that the chemical mechanism of biotin carboxylase involves the reaction of bicarbonate and ATP to form a carboxyphosphate intermediate, which either reacts with the enol (enolate tautomer) of biotin to form a tetrahedral adduct, or collapses into CO_2 and phosphate with subsequent attack of biotin on CO_2 (Waldrop *et al.*, 1994).

Konishi and Sasaki (1994) studied the localisation of two forms of ACC by comparing extracts produced from leaves and plastids. The results suggested that there were two sites of malonyl CoA biosynthesis in plants, a prokaryotic form in the plastids, and a eukaryotic form that was likely to be found in the cytosol. These two forms had differential tolerances towards herbicides.

Page *et al.* (1994) showed that ACC exerts strong flux control over lipid synthesis in barley and maize leaves. They determined apparent flux control coefficients of 0.58 and 0.52 for this enzyme in barley and maize respectively. The metabolic control theory was the basis of the work and assumes that control is shared amongst all enzymes of a metabolic pathway. The flux control coefficient measures the degree of flux control exerted by a step in a pathway, therefore an enzyme with a greater value exerts a greater influence over the regulation of the pathway than that with a lower value.

Termination of the synthesis of fatty acids is catalysed by the activity of an acyl-ACP-thioesterase once the chain has reached a chain length of C16 or C18 (Slabas *et al.*, 1994).

Fatty acids form the building blocks of a number of different types of lipids. The esterification of free fatty acids to glycerol to form triacylglycerol results in a group known as the neutral lipids. Synthesis of triglycerides involves the expenditure of energy and L-α-glycerol-P, which is derived from dihydroxyacetone-P, one of the intermediates of glycolysis. Two fatty acyl-CoA molecules condense with the L-α-glycerophosphate to form phosphatidic acid. A phosphate group is released upon hydrolysis with phosphatase resulting in the formation of a diglyceride,which condenses with another fatty acyl-CoA to form the triglyceride (Larner, 1971).

The *Fungi Imperfecti*, to which *P. expansum* belongs, generally have a lipid content that is 6-7% of their dry weight. This includes mainly 16:1, 18:2 and 18:3 fatty acids. There is a differential distribution between the triglyceride and polar fraction. The triglycerides, which store fatty acids tend to contain 16:0 and 18:1, whereas the polar lipids contain 16:1, 18:2 and 18:3 fatty acids (Shaw, 1966).

Polyunsaturated fatty acids (PUFAs) are found in almost every higher protist and do not generally occur in bacteria. They are defined as fatty acids containing two or more double bonds in the molecule. The most common is the 18 carbon linoleic acid, 18:2 (9,12). Higher protists like fungi tend to contain higher proportions of PUFAs in their lipids than in those found in higher animals and plants (Shaw, 1966).

Fungi are known to have a high content of PUFAs, despite fatty acids being synthesised saturated. Desaturase activity is confined to microsomes and in *N. crassa* there is an absolute requirement for acyl CoA, O_2 and reduced nucleotide. Most Mixed Function Oxidases (MFOs) have an absolute requirement for NADPH, but the *N. crassa* enzymes preferred NADH. Once acyl CoA is bound it can serve as a substrate in a number of reactions: (1) nucleotide-O_2 dependent desaturation; (2) phospholipid formation; (3) triglyceride synthesis; and (4) Deacylation. Acyl CoA is bound tightly to membranes so that it is available to all 4 groups of enzyme activities without having to pass through an unbound state (Baker and Lynen, 1971).

Bloch *et al.* (1961) reviewed the biosynthesis and metabolism of unsaturated fatty acids. Desaturation is a process that requires O_2 directly, and not a straight

dehydrogenation, as demonstrated by the requirement for unsaturated fatty acids by fungi grown under anaerobic conditions. Extraction of a cell free system from yeast showed that the desaturase activity was particle bound, and that CoA esters of fatty acids acted as substrates. O_2 and NADPH are essential for this process. The involvement of a flavoprotein-linked dehydrogenase was discounted since artificial electron acceptors like phenazine methosulfate and methylene blue could not be substituted for O_2. O_2 acts as the immediate electron or hydrogen acceptor without becoming covalently linked to the substrate. Further desaturation of the monounsaturated fatty acid, oleic acid also required an O_2 atmosphere, suggesting a similar process occurred for polyunsaturation.

In animals desaturation of fatty acids is carried out by microsomal enzymes in the liver. The system involves NADH-cytochrome b_5 reductase, cytochrome b_5, and an O_2-dependent desaturase containing a single non-heme Fe atom. NADH reduces oxidised cytochrome b_5 reductase, which is reoxidised by reduction of 2 cytochrome b_5s (the Fe ion goes to the 3^+ state) with the release of 2 H^+. The cytochrome b_5 is then oxidised by the desaturase by the dehydrogenation of stearoyl CoA, comcomitant with the formation of H_2O from the O_2 terminal electron acceptor.

The fatty acyl CoA desaturase system of yeast microsomes also involved cytochrome b_5 as an electron carrier (Tamura *et al.*, 1976). The terminal desaturase in this system was sensitive to relatively high concentrations of cyanide. The reducing equivalents for the desaturation reaction are supplied from the reduced form of cytochrome b_5. The reductase forms an essential part of this reaction. The rate of reoxidation of the cytochrome was stimulated by the addition of palmitoyl CoA.

Aerobic desaturases are multienzymic mixed function oxidases (MFOs), and there has been some controversy over whether the substrates are unbound acyl CoA (or ACP), enzyme bound acyl, or phospholipids. GLC analysis of microsomal fatty acids extracted from *Neurospora crassa* mycelium showed 80 % of these were 18C, and of these 90 % were unsaturated, with linoleic acid being a major component. Oleyl phospholipid was an intermediate in the conversion of oleyl CoA to linoleyl phospholipid (Baker and Lynen, 1971).

Meyer and Bloch (1963) observed that *Torulopsis utilis* cells grown at suboptimal temperatures contained a much greater proportion of more highly unsaturated fatty acids. The increase in desaturating enzyme activity was due to an increase in activity in the particulate fraction and not due to increased temperature sensitivity of the desaturase system.

In *P. chrysogenum*, endogenous fatty acids were elongated to C_{18} with acetate and desaturated in a sequential pattern: stearate → oleate → linoleate → linolenate. Myristic acid had a role as an intermediate molecule, but has an inferior capacity to form an enzyme bound intermediate required of a precursor for elongation and desaturation (Bennett and Quackenbush, 1969).

Experiments as long ago as 1935 (Prill *et al.*, 1935) have shown that the amount and nature of fat produced by fungi can be affected by a number of factors which include initial glucose concentration, ammonium nitrate concentration, pH, temperature and aeration. High fat contents were particularly favoured by a neutral or slightly alkaline medium containing high initial glucose and low ammonium nitrate levels.

The role of O_2 in the sterol biosynthetic pathway has been established previously (Hampton *et al.*, 1996). The expression of both isozymes of HMG CoA reductase (HMGR), Hmg1p and Hmg2p, are regulated by oxygen. Heme is the molecular signal for the contrregulation (i.e. opposite regulation of two isozymes by the same stimulus) of these isozymes. Heme stimulation of the *HMG1* gene is mediated by the Hap1p transcriptional activator, which activates numerous other O_2-stimulated genes. The O_2-dependent transcriptional repressor Rox1p appears to have no role in the repression of the *HMG2* gene despite the presence of a Rox1p-binding consensus sequence in the *HMG2* promoter. The net effect is that during aerobiosis the predominant isoform is Hmg1p, and in anaerobiosis, the predominant isoform switches from Hmg1p to Hmg2p.

O_2 also plays a role in the late mevalonic pathway. It is required for the epoxidation of squalene to squalene epoxide, which is then cyclised to lanosterol. This is

converted to ergosterol in fungi. The pathway prior to this does not require O_2 at all. The availability of O_2 is a critical determinant of the flow of molecules through the mevalonate pathway.

Losel (1990) reviewed the important role of lipids in the structure and function of fungal membranes. Lipids make up 30-50 % of the fungal membrane content. The compartments of the cells contain varying amounts of lipids. For example, the tonoplast contains more phospholipid than the plasmalemma, which contained a higher proportion of neutral lipid. 25 % was sterol, versus 6 % in the tonoplast. There are also significant chemotaxonomic correlations in membrane lipids. Membranes are composed of lipid bilayers within which proteins/enzymes are anchored at varying depths. Phospholipids, sphingolipids, sterols and glycolipids are major components of membrane lipids. The presence of double bonds in the fatty acids of the phospholipid affects the fluidity of the membrane. Sterols contribute to the stability of the membrane by locking the shape across fatty acid chains of polar lipids. The role of triglycerides in membranes is unclear. They may lie between the phospholipid layers forming lipid bodies, or provide reserves of acyl lipids for further membrane development.

Phospholipids form the main structure of the fungal membrane, of which phosphatidyl choline and phosphatidyl ethanolamine are the two most common types. Fungi have been shown to respond to changes in temperature and other environmental factors by changing the length or unsaturation of the fatty acid chains to maintain membrane function. Ergosterol is the most predominant sterol in higher fungal membranes. The sterols play a structural role in membranes, some of which play special regulatory or morphogenetic role. Sterol esters varying in amounts during the fungal lifecycle and are a storage form of sterols and fatty acids. Carotenoids, glycolipids and sphingolipids are also present in fungal membranes.

Membrane lipids are influenced by environmental and nutritional factors. At lower temperatures the fatty acids are present at higher degrees of unsaturation. The sterol and carotene content changes with growth at different temperatures. O_2 is required for

fatty acid desaturation and for sterol synthesis. Growth in strictly anaerobic conditions requires a substrate sterol and unsaturated fatty acids.

Suutari (1995) showed that fatty acid unsaturation increased in *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma reesei* when the growth temperature was lowered. In *P. chrysogenum* the linolenic acid content increased concomitant with a more pronounced decrease in the less saturated fatty acids oleic acid, palmitic and linoleic acids. The change in mycelial fatty acid profiles at varying environmental temperatures did not linearly correlate with changes to O_2 solubility or growth rate. Studies with the yeast *Candida utilis* showed that growth temperature and DO tension were separate effects. Generally, lipid accumulation is related to growth conditions restricting protein biosynthesis and was one of the factors defined in the post-growth transition phase defined by Borrow *et al.* (1961, 1964). This same mechanism could operate under changes in temperature. Although the factors that determine the fatty acid profile of polar and neutral lipids are known to differ between fungal species, the sum of temperature-induced changes in the lipids were similar in the fungi studied, supporting the presence of common regulatory mechanisms.

Bloomfield and Bloch (1960) studied the formation of Δ^9-unsaturated fatty acids in *S. cerevisiae*. This required O_2, NADPH and both the soluble and particulate fractions of cells. The oxygen requirement was absolute, desaturation would not occur when this was replaced by artificial electron acceptors. The experiments confirmed that desaturation is oxidative and not a dehydrogenation. Palmitic acid only underwent desaturation in the form of the CoA thioester, indicating that carboxyl activation was a prerequisite for biological transformations of a fatty acid. The intracellular site of desaturation could not be shown by these experiments. Both the heavy and light fraction at 8000*g* converted palmitic to palmitoleic acid. This indicates that there is possibly more than 1 cytoplasmic constituent of yeast required in this process and that this was independent of the cytochrome system.

Mammalian experiments have shown that the non-esterified fatty acids of serum rise in metabolic states in which NADPH is limiting (starvation). A decrease in the available NADPH would reduce the formation of unsaturated fatty acids (of which at

least one is required for the complete synthesis of a triglyceride molecule), curtailing triglyceride synthesis, causing an accumulation of unesterified fatty acids. (Bloomfield and Bloch, 1960)

7.1.1 Objectives

Experiments to investigate lipid metabolism in *P. expansum* sought to answer two questions:

1. Did agitation have an effect on the accumulation of total lipid in *P. expansum*?
2. Could agitation affect the degree of saturation of lipids?

The extraction solvent (Petroleum ether) primarily isolated neutral lipids, into which the triacyl glycerides and sterols fall. No attempts were made to specifically quantify the unsaturated lipids - the analysis was purely qualitative.

Some initial investigations into the effect of agitation on sterol biosynthesis in *P. expansum* were undertaken as part of this work. These were abandoned because of the variability of results obtained and problems experienced in performing the perchloric acid-vanillin assay (Few, 1965). It was hoped that this would contribute to a greater picture of the fate of acetate in this organism.

7.2 MATERIALS & METHODS

7.2.1 Culture Conditions.

Please refer to Chapter 3.

7.2.2 Neutral Lipid Extraction and Quantitation.

A soxhlet was used to extract neutral lipids from freeze-dried mycelium using diethyl ether as the solvent. Samples were ground to a fine powder in liquid nitrogen, placed into Whatman extraction thimbles, and the weights recorded in order to provide comparisons between samples of the lipid weight extracted on a dry weight basis. The soxhlet was run for a period of 8 hours, allowed to cool, and the solvent extract collected in preweighed round bottom flasks used for the extraction. The solvent was removed using a rotary evaporator. The lipid residue remaining was weighed to give the total accumulated neutral lipid of the sample.

7.2.3 Thin Layer Chromatography of Neutral Lipids.

The extracts produced for neutral lipid quantification were dissolved in 1 mL diethyl ether for TLC analysis. Silica gel G glass plates (Schleicher and Schnell) were heat activated prior to use. Lipid extract (10 μL) was spotted onto plates and run in 90:10:1 v/v/v petroleum ether: diethyl ether: acetic acid in a system employed by Mangold (1969). The petroleum ether in the solvent was used to fractionate alcohols, aldehydes, mono-, di- and triglycerides, while weakly polar lipids stayed at the solvent front. The acetic acid was included to prevent streak and tail formation by free fatty acids in the resulting chromatograms.

Initially two developing systems were used. A solution of 50 % H_2SO_4 was sprayed onto plates and heated to give purple/crimson spots. This solvent visualised all lipids. The second system, adopted for most of this work, was I_2 vapour, which was used to visualise unsaturated lipids, N-containing lipids and only some saturated lipids.

7.3 RESULTS

7.3.1 Quantitative Analysis of Non-Polar Total Lipids.

The quantitation of diethyl ether-extractable lipids is shown in Figures 54 and 55. The ME lipid weights per gram of dry mycelium were generally greater than those extracted from the YEGB medium. Lipid accumulation in the ME cultures lagged 24h, but was the same as the shake cultures at 48h. The shake cultures continued to accumulate lipid, whereas the static cultures decreased gradually.

Fatty acid synthesis and utilisation can occur simultaneously, and therefore it must be assumed that increases or decreases in the mycelial lipids represents a change in the balance between these processes rather than either one or the other occurring in isolation.

The lipid weights did not drop below approximately 30 mg/g dry weight (except at 24h in the ME static cultures) in cultures grown in both media. This may represent the minimum level of lipid in this organism at which accumulation and depletion are balanced, however the cultures were not grown over a long period and this conclusion may be inaccurate.

The growth phases defined by Borrow *et al.* (1961, 1964) include an accumulation phase prior to idiophase during which lipid accumulation occurs just before the exhaustion of medium nutrients. A period of lipid accumulation occurred in *P. expansum* after 48h in shake cultures grown in the ME medium. This coincided with depletion of medium nutrients found in Chapter 4 (carbohydrate) and 6 (ammonium). This effect was not observed in the static cultures grown in the same medium. The lower levels of lipids in the static cultures of this medium reflected the slow nutrient uptake by *P. expansum*, and the onset of secondary metabolism earlier than the shake cultures (Chapter 5). The generation of greater amounts of acetate in the shake cultures would also lead to a greater amount of this flowing into fatty acid biosynthesis, particularly if secondary metabolism enzymes were not active or the flux through these was low.

Figure 55 shows that the pattern of lipid accumulation in shake cultures of *P. expansum* grown in YE medium differed to ME shake cultures. Over the first 24h the mycelial lipid weights were similar for shake and static cultures. The static culture continued to accumulate lipid to 48h and then decreased to approximately 40 mg lipid/g dry mycelium. The shake culture only gradually increased accumulated lipid levels after 48h. The decrease in lipid levels after 48h in the static cultures indicated that the organism was degrading fatty acids for use in other areas of metabolism. This time coincided with the depletion of medium nutrients (Chapters 4 and 6) and a substantial increase in patulin production (Chapter 5). The breakdown of lipid could serve to provide acetate for polyketide production, which was fully operational at this time. These possibilities are considered further in the Concluding Discussion (Chapter 8).

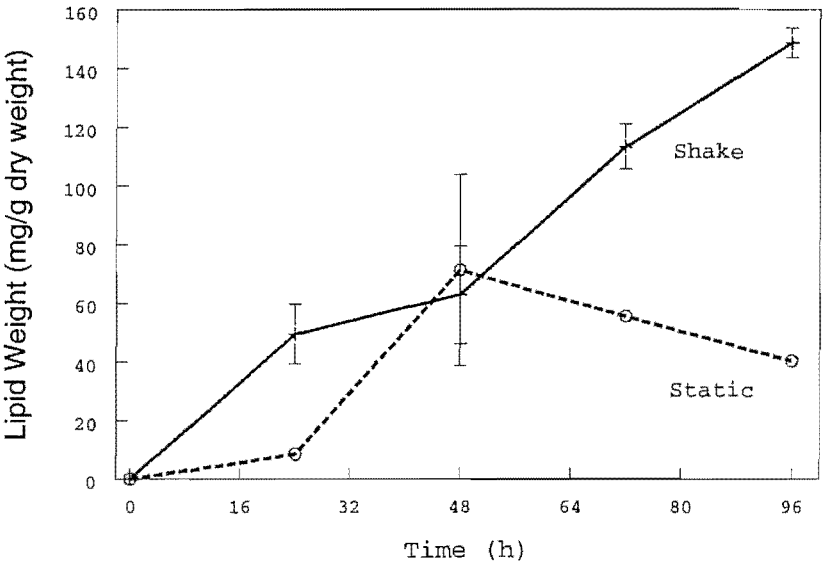


Figure 54. Mycelial lipid accumulation in *P. expansum* grown in ME medium.

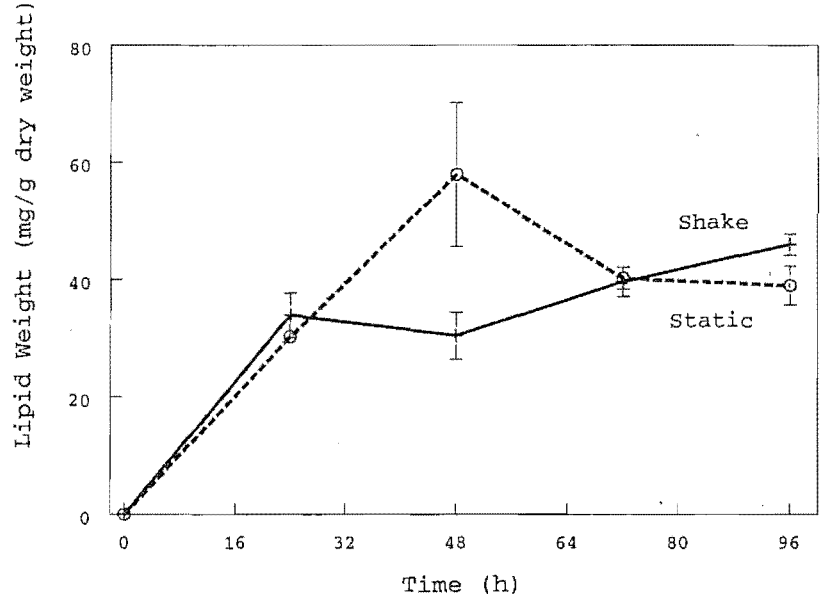


Figure 55. Mycelial lipid accumulation in *P. expansum* grown in YE medium.

7.3.2 Qualitative Analysis of Unsaturated lipids.

Thin layer chromatography with I_2 vapour visualisation was used to study the unsaturated lipids present in diethyl ether extracts of freeze-dried *P. expansum* mycelium. The degree of unsaturation of fatty acids is related to the availability of O_2 since the desaturase reaction is catalysed by Mixed Function Oxygenases, and this was studied in shake and static mycelial lipid extracts.

Table 16 shows the R_f values for spots commonly found in chromatograms of mycelial lipid extracts. The extra spot present in the ME extracts compared to the YE extracts was found at the second "solvent front" found on these chromatograms. This "solvent front was a line appearing on the plates at $R_f = 0.24$. Some spots resolved poorly. The spot giving the R_f values 0.48-0.59 was usually a streak, which ran to slightly different positions. The size of this streak suggested that this lipid was present in extracts in significant amounts. Identification of the spots was not pursued, although this could have been done using gas chromatography (GC). The only tentative identification that could be made was ergosterol, which ran at the same place as the spot at $R_f = 0.20$.

Table 16. Common R_f values for Thin Layer Chromatograms of Mycelial Lipid extracts of both shake and static cultures.

Rf	ME Medium	YE Medium
1.00	+	+
0.90	+	+
0.48-0.59	+	+
0.32	+	+
0.24	+	-
0.16-0.20	+	+

(+ indicates spot present)

Some differences were observed between the shake and static cultures of both media (not shown on Table 16). A compound giving a spot that ran at $R_f = 0.70$ was present in shake cultures that were older than 24h for *P. expansum* extracts from both media, whereas it was only present in the 24h static extracts.

Other spots appeared inconsistently on chromatograms, not occurring in repeated experiments, and at variable times, including spots at R_f 0.39 in YE shake 2 and static 1 cultures, and at $R_f = 0.82$ in shake 1 and 4 of both media, but not any of the other cultures.

The difference between shake and static was not therefore great since the only consistent difference was the R_f 0.70 spot, and the difference between the two media was the R_f 0.24 spot present in the ME medium. This was unexpected, although fungi tend to have a high content of PUFAs (Baker and Lynen, 1971), therefore the desaturase reaction may already have been inactive in the static cultures, with only a small specific increase in activity to generate the R_f 0.70 spot.

7.4 GENERAL CONCLUSIONS

Fatty acid production was affected by agitation, however the main quantitative differences were seen between the ME and YE media. The degree of neutral lipid accumulation was similar within a particular type of medium, although agitation affected timing of accumulation and whether or not the accumulation was on-going. The decrease in ME static culture lipid accumulation indicated that the fatty acids were being broken down to in order to sustain metabolic turnover in the fungus, or that breakdown was occurring at the same rate as the shake cultures but the acetate precursors for synthesis were being diverted into polyketide synthesis.

In the YE medium, the start of FA degradation appeared to occur at the same time as the exhaustion of carbohydrate and ammonium (Chapter 4 and 6).

There were not many qualitative differences in the unsaturated fatty acids being monitored by thin layer chromatography. There was an extra spot in the ME extracts at $R_f = 0.24$ which was not present in the YE extracts, indicating that the nutritional state of the organism had a small effect on this parameter. There was also a streak between $R_f = 0.48 - 0.59$, which varied in length and intensity, possibly indicating that it was present in varying amounts. A standard consistent method was used to extract the lipids so that valid comparisons could be drawn, and therefore further work using gas chromatography could have been performed on lipids giving rise to single spots, which gave varying intensities, for their quantitation and identification.

There was an extra spot present at $R_f = 0.70$ in shake cultures of both media, but this was the only consistently produced shake spot. The differences between media types appeared to be greater than the differences between shake and static.

There was a definite correlation between O_2 concentration (shake) and increased lipid production in the ME cultures. Mechanisms that would allow this to occur include increased NADPH for the reductive biosynthesis of fatty acid through increased pentose pathway activity. Citrate accumulation is also a key fatty acid biosynthesis regulator. Increased fatty acid synthesis could reflect an increased flux through the

TCA cycle and the accumulation of intermediates. These potential regulatory factors are discussed further in the Concluding Discussion (Chapter 8).

Agitation appeared to have a slightly negative effect on lipid accumulation in the YEGB medium. If the accumulation of lipid/fatty acid production is indeed related to activities such as the TCA cycle, a strong possibility exists that catabolite repression may have prevented accumulation of the citrate effector, and therefore lower levels of lipid synthesis and accumulation are observed. Once again, this is discussed further in Chapter 8.

Further work could have been done to identify the lipids that featured in the changes in the unsaturated profiles, involving GC-MS and radiogas chromatography. However, since the changes weren't numerous, this wasn't a priority. The effect of agitation on other classes of lipids could also have been investigated, in particular changes to the lipid membrane composition with agitation could have provided some useful insights into the lipid metabolism of this organism.

CHAPTER 8

CONCLUDING DISCUSSION

A series of experiments were conducted to investigate different areas of the metabolism of *Penicillium expansum* in an attempt to determine the effect of agitation, and therefore aeration on secondary metabolism in this organism. The results presented demonstrate that agitation affects numerous pathways and the growth habit of this fungus, in both the ME and YEGB media used.

8.1 GROWTH AND MORPHOLOGY OF *P. EXPANSUM*.

The effect of agitation on the rate of growth was demonstrated in the experiments to measure dry weights of mycelium. The initial growth period (up to 24 h) was enhanced in shake cultures of both media. However shake cultures, grown in the ME medium, decreased in growth rate significantly as the nutrients were exhausted. The slowing in growth of the YE shake cultures was probably not for the same reason and was due possibly to the volume constraints of the culture medium. The YE shake cultures grew as thick clumps of mycelial suspensions with some pelleting occurring. The limited space in which the fungus had to grow probably gave rise to concentrated areas of growth and aggregation, which would cause pellet formation by the agglomeration of hyphal elements; one of three mechanisms defined by Nielsen (1996) for microbial pellet formation. The clumping in the YE shake cultures changed the viscosity of the culture, behaving as non-Newtonian fluids. This was likely to be due to the formation of a loose three-dimensional structure from the branched mycelial network, which would give the suspension rigidity and a yield stress (Banks, 1977).

The pelleting observed in the ME shake cultures was likely to have been due to the nutrient status of the medium. The conidial concentration in the inoculum was

unlikely to have been a factor in this because all cultures received the same size of inoculum.

Static cultures grew as surface mats in both media, the YE culture formed a thick, heavily sporulating mat by 72h.

Shake and static conditions represent two different mechanisms for the introduction of O_2 to fungal cultures. The turbulence caused by agitation reduces the films around cells that form some resistance to the movement of O_2 into cells, as well as resistances caused by the gas-liquid interfaces. By contrast shake cultures create a situation where the same "face" of mycelium contacts both the O_2 phase and the culture medium. Depletion of nutrients occurs at an equivalent rate over all parts of the mycelium, except where pelleting has occurred. This is relevant to shake cultures, which grow in a more homogeneous manner than static cultures.

Static surface cultures rely on diffusion for the introduction of O_2 to the medium. This allows the formation of a gradient of O_2 through the surface of the medium and localised DO gradients around the cells. Similarly, the lack of mixing in the static medium causes the formation of nutrient gradients around cells also. This sets up a situation in which opposite faces of the mycelial mat are exposed to different levels of nutrients and O_2. One is nutrient rich, but lacking in dissolved O_2. The other is nutrient poor and, although at the gas interface, has access only to the DO present in the upper layer of the culture medium. It is likely that dissection of the mat to separate the surfaces would have revealed different metabolic states. However, this would be very difficult to achieve, particularly in the younger cultures with finer mats.

As discussed earlier in this chapter, pelleting is likely to have arisen through two different mechanisms in the two different media. The environment in which the pellets were formed also gave rise to different abilities of O_2 to be transferred through the media. The YE shake pellets were formed through the aggregation of hyphal elements in a thick suspension. It is likely that the DO levels in the suspension were significantly reduced at 72h and 96h compared to earlier since the viscosity change would prevent effective mixing of the mycelium and the culture medium from occurring. The ME pellets were likely to have formed due to the low nutrient levels

in the medium (Phillips, 1966) and were formed independent of hyphal agglomeration and clumping. ME pellets allowed the culture to grow in an environment with low viscosity and high aeration efficiency.

Pellets also have gradients of nutrients and DO from the surface to the interior. The internal environment lacks in both O_2 and nutrients whereas the outer face has access to both. Once again, there would have been different metabolic states present at different levels in the pellets, which would have been difficult to isolate and study.

The presence of more than one metabolic state would influence the bulk analysis of mycelial parameters undertaken in this work. In particular, the low DO and increased viscosity of the YE culture would probably be responsible for the closeness of shake and static results for a number of parameters. This is discussed later in this chapter.

Another consideration in the ability for DO transfer in the respective media is ionic strength. This is greater in the YEGB medium than the ME medium, although unlikely to be at such a level that would cause problems. Hansberg and Aguirre (1990) state that an increase in ionic strength results in decreased O_2 solubility. Therefore the YE medium would have been more likely to have been affected by this. DO was not measured in any experiments and therefore statements to this effect are merely speculation. It is also likely that any decrease in the DO levels would be circumvented by the increase in available carbon in the YE medium which would help drive metabolic reactions. The ionic strength is not likely to limit the O_2 concentration in the medium, however the effects are more likely to manifest in the coalescence of bubbles in the medium (Zieminski and Whittemore, 1971).

The YE medium had significantly greater levels of carbohydrate than the ME medium. Initial reducing sugar concentrations were 6-fold greater in the YE medium. The growth of the YE cultures was limited at 96h, despite 21 mM of reducing sugars remaining. Therefore some other growth limiting mechanism or inhibition of sugar uptake must have been occurring in order for this remaining reducing sugar not to be utilised.

The transport of sugars into fungal cells is likely to be a combination of active and passive systems. A slowing of metabolism would result in less ATP being produced that could be utilised in active transport. This could explain the decrease in growth rate late in the YE shake cultures, since metabolism would be switching away from primary pathways into secondary metabolism. It was noted that the patulin pathway was not important did not appear to be functional throughout the YE shake cultures.

The relationship of ATP generation to sugar uptake could easily account for the slow utilisation of reducing sugar in the ME cultures, particularly the static experiments since the reducing sugar concentration in ME static culture filtrates decreased at a very slow rate. There was a general trend for low enzyme activities in the primary metabolic pathways monitored in the ME static cultures, which is reflected in the growth rate, which is indicative of a reduced ability to generate ATP.

The introduction of O_2 in the shake cultures and its relationship to ATP generation would seem the most likely explanation for the utilisation of reducing sugar and growth from the outset in these cultures compared to static cultures. Another possibility for the lag in the static cultures growth and reducing sugar utilisation could also be due to inducible systems. It is possible that sugar transport in the shake cultures is induced immediately though the increased activity of the metabolic pathways and therefore the generation of an appropriate metabolic signal, which occurs approximately 24h later in the static cultures.

8.2 RESPIRATION.

The rate of sugar uptake did not appear to be related to glycolytic activity in this organism. Glucokinase activities were not affected significantly by agitation, since shake and static activities were similar in both ME and YE media. The ME shake glucokinase activity correlated well with the reducing sugar uptake, however the same conclusion could not be drawn for this activity in the other cultures. Therefore it was concluded that the same amount of active glucokinase is available under both aeration conditions, and is therefore not regulated by aeration.

Fructokinase, the second hexokinase activity monitored, did show differences between shake and static cultures in the YE medium, but not in the ME medium. The ME fructokinase activities were low and the static level possibly reflects a base rate that is maintained by the organism. It is possible that metabolites are fed into glycolysis through this enzyme at a constant rate. The activities of fructokinase and glucokinase did not correlate and they appeared to operate independently from each other.

The YE fructokinase activity varied for shake and static cultures and it may be significant to note that the increase or decrease in activity matched increases and decreases in the YE mannitol dehydrogenase activities. Both these enzymes are components of the mannitol cycle proposed by Hult *et al.* (1980), and if this cycle was operating in this organism, its presence could be verified by this apparent relationship. Fructokinase and mannitol dehydrogenase are similar because of the proximity of these two enzymes to the main glycolytic pathway (Figure). Mannitol dehydrogenase is involved in the metabolism of polyols, which are the storage form of carbohydrates in many filamentous fungi, therefore it follows that its activity reflects events in the main primary pathways. The lack of relationship in the ME medium is likely to be due to poorer nutritional state of this medium compared to the YE medium, which would encourage the storage of carbohydrates.

The Tricarboxylic Acid (TCA) Cycle forms the second major pathway in respiratory metabolism in aerobic organisms and the NAD^+-utilising form of isocitrate dehydrogenase (ICDH) was monitored to indicate activity in this cycle. The TCA cycle was prevalent in early shake cultures in particular in both media. This would be a direct effect of agitation since there would be plenty of O_2 available to perform its role as the terminal electron receptor in the generation of ATP in oxidative phosphorylation, which picks up the electrons released from the TCA cycle. The levels of this enzyme in static cultures were insignificant.

In the YE medium, both shake and static cultures peaked early in the levels of NAD^+-ICDH and then decreased. The lack of terminal O_2 would have much less effect in this medium because a greater level of substrates would be available for utilisation through alternative pathways. In particular, the level of TCA metabolites is

influenced by the feed and bleed-off to amino acids, etc. through transamination. Fatty acid biosynthesis is the major alternative route for the utilisation of acetyl CoA, which feeds from the TCA cycle. The relationship between the TCA cycle and fatty acid synthesis is covered later in this discussion in Section 8.6.

8.3 NADPH GENERATION.

The level of NADPH was considered to be a major factor in determining the fate of acetate in the metabolism of *P. expansum*. The means by which this is generated have been reviewed in Chapter 4, and each of the pathways for its generation were considered carefully for their potential to contribute to the cellular NADPH pool, and therefore their ability to effect flux towards lipid versus polyketide synthesis.

The activity of the pentose phosphate pathway in *P. expansum* was affected by aeration, particularly in the ME medium. Glucose-6-phosphate dehydrogenase (G6PD) activity was significantly higher than static activities at 48-72h in cultures grown in ME medium. This was correlated with the presence of two extra isozymes at this time in these cultures. The formation of extra isozymes is a means for regulation of G6PD. Determination of the K_m of crude extracts of 72h shake and static ME cultures revealed a greater affinity for the substrates, glucose-6-phosphate and $NADP^+$ in the shake extracts. Potential inhibitors were tested and strong inhibition was shown by the reaction products 6-phosphogluconate and NADPH, indicating that these compounds are likely to be competitive, allosteric inhibitors of the ME G6PD. Glutamine, NH_4^+, ATP, AMP and erythrose-4-P were also likely allosteric inhibitors, the latter providing a means for feedback inhibition of the pathway since it is a product in the second non-oxidative portion of the pentose phosphate pathway.

A series of experiments omitting the cofactor Mg^{2+} from the assay were performed to establish whether an interaction between ATP and Mg^{2+} could occur, which could result in the withdrawal of available Mg^{2+} from the enzyme activity. However there were no significant differences in activity observed in these experiments except at 96h in the ME cultures. In this particular medium the formation of ATP-Mg^{2+} complexes

could be a potential regulatory mechanism since it is not supplemented with trace metals like the YE medium.

The 6-phosphogluconate dehydrogenase (6PGD) activity indicated that this enzyme was probably responsible for maintaining equilibrium between 6-phosphogluconate and glucose-6-phosphate. Under conditions favouring glycolysis, a specific level of G6P would need to be maintained for use in the glycolytic pathway. The maintenance of glucose-6-P levels would be more favoured in the ME medium, but not the in the YE medium since a greater flux to 6PG would be required to ensure that glucose-6-P levels were reduced. Increased levels of 6PGD would allow the passage of more substrate through the pathway.

The contribution of each of the proposed NADPH generators is best assessed in the ME medium where clearer relationships between the different parts of metabolism could be observed. The sub-cellular location of each of these enzymes/pathways is an important factor in determining its ability to influence metabolic pathways such as fatty acid biosynthesis, which is cytoplasmic. Similarly each of the potential NADPH generators assessed in this work were also cytoplasmic. With respect to the relative levels of activity of each of the enzymes, and the apparent relationship between these activities and fatty acid biosynthesis, it was concluded that the pentose phosphate pathway would make the greatest contribution to the cytoplasmic NADPH pool. This is consistent with the role of this pathway as the major NADPH generator in the metabolism of most cells. Malic enzyme and the $NADP^+$-utilising form of isocitrate dehydrogenase would make only minor contributions based on the moderate activities observed in these experiments. The mannitol cycle was unlikely to be occurring in this organism since levels of mannitol dehydrogenase were very low, and therefore would not have any effect on NADPH concentrations.

The presence of two additional G6PD isozymes in the 48-72h shake ME cultures correlated with higher levels of this activity. The production of extra isozymes is a potential regulatory mechanism, particularly if these impart differing substrate affinities to the activity. This was confirmed when the K_m of $NADP^+$ and glucose-6-phosphate were determined to be lower (meaning the enzymes had greater affinities for them) in the shake culture extracts than in the static ones.

No such difference in G6PD activities and isozyme patterns were observed for the YE extracts. The activities were similar and the same three isozymes were present in both shake and static cultures grown in this medium. It is possible that the rapid uptake of sugar in the YE cultures may have lead to catabolite repression, resulting in similar activities between shake and static, since by the second day of cultures the static cultures had caught up to shake cultures in the rate of glucose utilisation.

Further conclusions regarding the regulation of G6PD, particularly in the ME cultures were able to be drawn from experiments assessing the inhibitory effects of a number of compounds. Significant inhibition was observed with the reaction products 6-phosphogluconate and NADPH, and it is likely these effect competitive and allosteric inhibition of the enzyme. Inhibition by glutamine, NH_4^+, ATP, AMP and erythrose-4-P was also likely to have been allosteric. The latter would also operate as a feedback inhibitor since it is a metabolite further down the pentose phosphate pathway from G6PD.

The possibility of an effect on activity by the interaction between ATP and the cofactor for G6PD, Mg^{2+} was also investigated. The omission of this ion from assays showed there was no real effect on the enzyme activity, therefore it was unlikely that ATP in the cellular extracts was interacting with the additions to the assay. The only point in time in the ME cultures when a small difference was observed was at 96h. This represents a time at which available ions would have been utilised by the fungus in this nutrient poor medium, which had not been supplemented with trace metals, as had the YEGB medium. This particular situation represents a further possible regulatory mechanism provided the nutrient medium is sufficiently deplete of metal ion cofactors. This would be more important in the natural growth environment of *P. expansum*, but much less likely on laboratory substrates.

The activity of the second enzyme of the pentose phosphate pathway, 6PGD, was also investigated in this work. It is likely that the activity of this enzyme maintains an equilibrium between the glucose-6-P and 6-PG of the G6PD which precedes it. Under this scheme, if glycolysis was favoured, the equilibrium would favour the maintenance of glucose-6-P levels since this is the branch from glycolysis of the PP

shunt. This shift in equilibrium would be more favoured in the ME medium, and can be correlated to the generally lower activities of this enzyme in this medium. By contrast, the excess carbohydrate present in the YEGB medium would result in higher amounts of metabolites in glycolysis, including glucose-6-P, and therefore the pentose phosphate enzymes (provided they are suitably activated) would change their equilibria to absorb the higher concentrations of this substrate. The increased levels of 6PGD in this medium reflect a requirement to channel more substrate through this pathway.

Very low levels of mannitol dehydrogenase were observed in cultures grown in both media. This indicates that the mannitol cycle, if it exists, does not operate in this organism. Similarly, the lack of this activity would suggest that polyol metabolism is not occurring. This would seem more believable in the ME medium, but possibly not in the YE medium, which contained significant amounts of carbohydrate. However, polyol metabolism may not be a feature of *P. expansum*. Had the mannitol dehydrogenase activities been greater, it would have been interesting to use Zn^{2+} ions to inhibit its activity to observe the effects this would have on the ability of this cycle to generate NADPH for biosynthesis (Niehaus and Dilts, 1982).

Of greater interest was the similarity of the ME static activities of mannitol dehydrogenase and fructokinase, and of the matching oscillations between these two enzymes in the YE static cultures. If this cycle does indeed occur, then the observed fluctuations of these two enzymes could be accounted for through their participation in it. If the cycle is not present, then the fact that fructokinase and mannitol dehydrogenase both feed from glycolysis. However, no similarities between the glucokinase (glycolytic) activities in these cultures and those of these two enzymes were observed.

A point of interest in these studies was that the activities of the ME culture malic enzyme were greater than the same enzyme extracted from YE cultures. This is opposite to what was observed for nearly all of the primary metabolic enzymes evaluated in this work. The patterns of activity of this enzyme strongly resembled the TCA enzyme monitored, NAD^+-isocitrate dehydrogenase. This is inevitably due to the close relationship of malic enzyme to the TCA cycle. Higher activities were

observed in the shake cultures (particularly in the ME medium in which the activities of this enzyme were more significant). This was due to the increased levels of terminal O_2 available to the TCA/electron transport chain, which could drive more substrate through the cycle, and therefore also through malic enzyme. This corroborated with observations in *R. toruloides* that malic enzyme was regulated by the substrate availability when there was a lack of substantial inhibitors (Evans and Ratledge, 1985), particularly since allosteric regulation would require a certain level of substrate to be present in order for the enzyme to be activated.

Malic enzyme could be detected on native PAGE gels, however overloading of the gels was required to achieve this. A single isozyme was detected for all cultures, which would suggest that the regulation is likely to be solely allosteric and dependent on substrate concentrations. An isozyme for the YE cultures could also be detected, despite the enzyme assays indicating that there was little or no activity. The conclusion that can be drawn from this is that the YE malic enzyme was strongly inhibited and thus not able to be detected in the enzyme assays. Catabolite repression seems an obvious candidate to explain why this enzyme would be inhibited in this particular medium. Presumably electrophoresis was able to separate this inhibitor from the enzyme, thus allowing it to be detected using this technique. Further, the fact that the inhibitor could possibly have been removed by electrophoresis leads to the possibility that it was proteinaceous, probably more highly charged than the enzyme and able to associate tightly to an appropriately oppositely-charged domain on the enzyme to regulate it by changing its conformation. It is possible that protein inhibitors (and even repressors) are synthesised in response to catabolite-sensitive genes (or by a mechanism that mediates the presence of excessive amounts of catabolites, such as a phosphorylation cascade for example), and are utilised for the global inactivation of a number of enzymes, of which malic enzyme would be one.

NADP-isocitrate dehydrogenase activities were reasonable but probably insufficient for the cellular requirements of NADPH for biosynthesis. There were not significant differences between shake and static in both culture media since error margins overlapped between the agitation conditions. Native PAGE gels of this enzyme were mostly unsuccessful, however on occasions a single isozyme band appeared to be present throughout YE cultures. Similar conclusions could therefore be drawn to

malic enzyme regarding the regulation of $NADP^+$-ICDH, although the reproducibility of these experiments was not adequate enough to consider this a definitive explanation.

The activities of the $NADP^+$-utilising cytoplasmic isoform did not reflect those for the mitochondrial, NAD^+ form. This is to be expected since the two forms are separated intracellularly. The activity of this enzyme is considered in relation to fatty acid synthesis in Section 8.6 of this discussion, because the proposed role of this enzyme is that it supplies NADPH for fatty acid biosynthesis.

Attempts were made to extract and quantify $NADP^+$/NADPH levels based on assays by Toledo *et al.* (1995) using glutathione reductase were unsuccessful and therefore abandoned. An indication of the overall balance of $NADP^+$/NADPH would have been useful in forming conclusions about the role of NADPH.

8.4 POLYKETIDE BIOSYNTHESIS.

Patulin biosynthesis was not important in the shake cultures grown in YEGB medium. From the outset only the early pathway metabolites, 6-MSA and *m*-hydroxybenzyl alcohol were present. Toluquinol, although absent at 24h was present at 48h and thought to be produced from *m*-cresol on the main patulin pathway by the action of an MFO, which would have been activated in order to synthesise this compound. This MFO and the one producing *m*-hydroxybenzaldehyde would compete for the *m*-cresol. It is likely that normally the main pathway MFO would have a greater affinity for this substrate, however under increased O_2 concentrations, the toluquinol MFO would increase its affinity for its substrate, as indicated in Figure 57. A general increase in the level of patulin synthesis occurred by 96h, however toluquinol disappeared. This correlates in timing with the thickening of the culture and the resulting decrease in DO levels. There was clearly a shift in flux away from the primary oxidative metabolism that was influenced strongly by the presence of O_2, into an alternate route directing acetate into polyketide biosynthesis. The MFO producing toluquinol also would have been affected by the decrease in O_2 concentration, since it

has a lower affinity for this than the *m*-hydroxybenzaldehyde MFO, therefore not able to compete for the substrate.

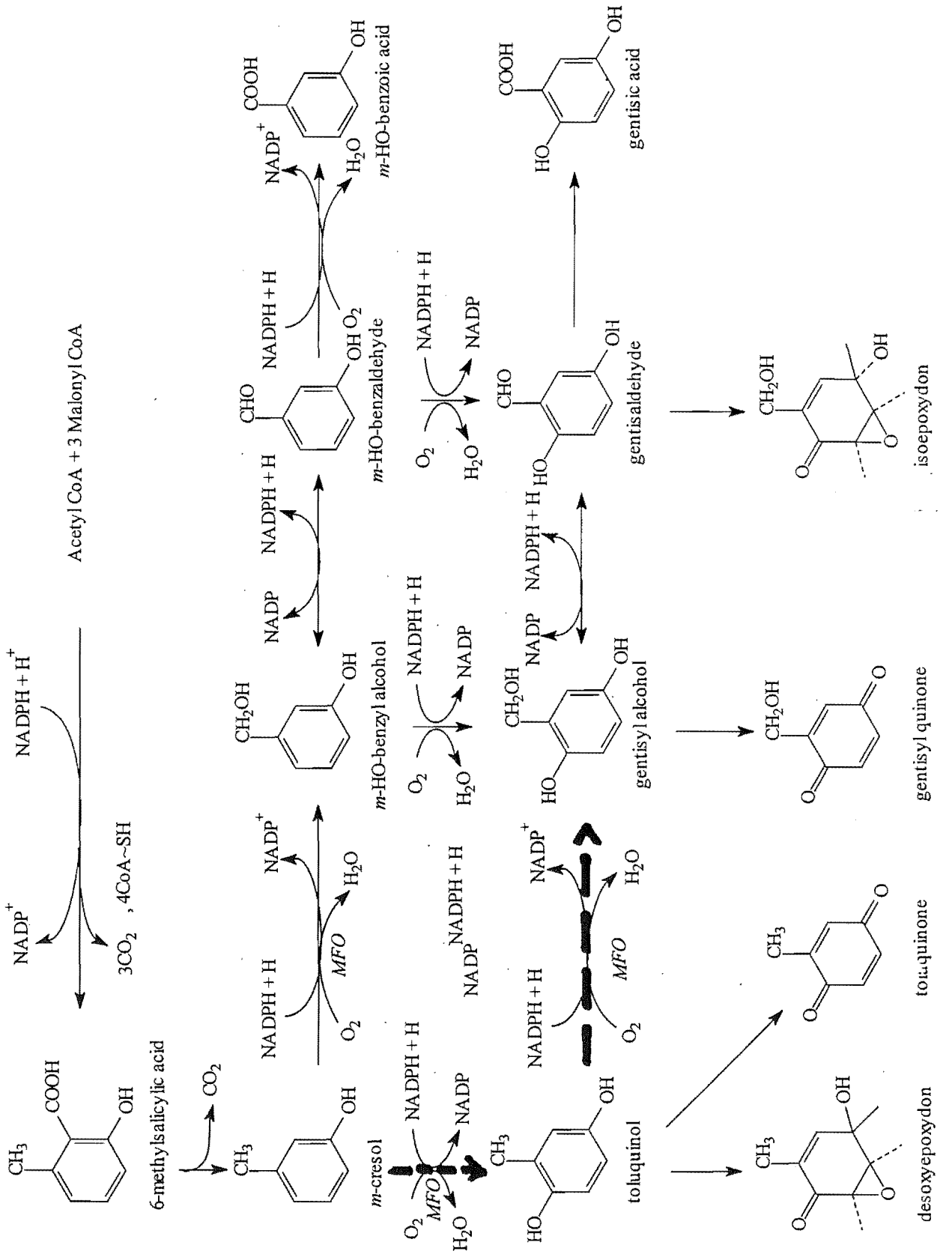
HPLC analysis showed 24h static YE cultures to have high levels of patulin production, however it is unlikely that patulin was produced in such high amounts at this time. The most probable explanation is that a precursor interfered in the measurement of patulin, eluting at a similar time from the stationary phase column.

Toluquinol was also produced in these cultures, which initially appears surprising if the explanation in the previous paragraph regarding its production in shake cultures is considered. However, the flux in the static cultures more strongly favoured polyketide synthesis and therefore there is more substrate for the MFOs to compete for. It is also likely that the DO levels at the surface, particularly in the early static cultures in which the mycelial mats were thin and sparse, would have been significant enough for the two MFOs to have been functioning.

The "yellow compound" present at day 1 in chromatograms of the ethyl acetate extracts of the cultures was assumed to be one of the late pathway metabolites occurring post-phyllostine. This was not present in any of the shake cultures, and was indicative of the general flux towards secondary metabolism in the YE static cultures.

The production of patulin in the YE static cultures became more dominant from day 3 onwards, coinciding with the thickening and differentiation of the mycelial mat. The relationship between sporulation and secondary metabolism has been noted previously and this is discussed in Chapter 2.1. A potential mechanism for the initiation of these processes mediated through the O_2 concentration is intracellular calcium. Calcium is a known regulator of cellular activities, mediated through interactions with calcium-binding proteins such as calmodulin to effect conformational changes in enzymes containing this protein, or to act as an effector on other enzymes to influence metabolism.

Figure 57. The early patulin biosynthetic pathway including proposed route during aeration (- - - -).



The relationship between Ca^{2+} and differentiation in fungi is well established. Griffin (1966) was one of the first workers to show that Ca^{2+} has a role in the differentiation of sporangia and gemmae in an isolate of *Achlya* sp. Hyphal extension and branching in *Fusarium graminearum* was also regulated by Ca^{2+} (Robson *et al.*, 1991). Decreased levels of Ca^{2+} increased the mean hyphal extension rate and growth unit length so that mycelia became more sparsely branched. This was antagonised by high concentrations of the calmodulin antagonists, trifluroperazime, W7 and chlorpromazine.

The transient rise in the intracellular Ca^{2+} concentration is the trigger for regulatory events mediated by Ca^{2+} interactions (Pitt and Kaile, 1990) with a variety of membrane-bound and soluble Ca-binding proteins. The membrane bound proteins are predominantly associated with the transport of this ion. The soluble proteins include calmodulin and tend to be the intracellular regulators. Calmodulin undergoes a conformational change upon Ca^{2+} binding, which allows it to interact with enzymes, proteins and peptides.

There are two main ways Ca^{2+} can elicit a cellular response via calmodulin (Pitt and Kaile, 1990). The first is amplitude modulation, where the magnitude of response is directly related to an increase in the concentration of Ca^{2+} in the cell and therefore the concentration of the Ca^{2+}-calmodulin complex. The second is sensitivity modulation, in which the concentration of the Ca^{2+}-calmodulin complex usually increases without a prior increase in intracellular Ca^{2+}. These two mechanisms tend to function co-operatively rather than independently.

Increases in intracellular calcium are mediated by the phosphoinositol cascade. Binding of a stimulatory factor to membrane receptors results in the activation of the enzyme phosphoinositase or phospholipase C, probably mediated by G-protein which cleaves phosphatidyl inositol 4,5-bisphosphate (PIP_2) to form the two intracellular messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Stryer, 1988). IP_3 acts on Ca^{2+} channels to release calcium into the cell, which can then interact with Ca^{2+} binding proteins such as calmodulin.

A direct linear relation was shown between phosphatidyl choline content, *in vivo* chitin synthase activity and mean hyphal extension rate by Binks *et al.* (1991) by varying the concentrations of choline chloride in the medium in which an *A. nidulans choc* choline auxotroph was grown.

Metabolic changes in the routes of carbohydrate metabolism based on glucuronate have been observed in *P. notatum* following sequential biphasic uptake of Ca^{2+} by the mycelium, and subsequent accumulation at subcellular sites (Pitt and Kaile, 1990). Pyruvate dehydrogenase (glycolysis) and oxoglutarate dehydrogenase (TCA) were both inactivated in Ca^{2+}-induced preparations of this fungus. ATP depletion and biosynthesis were also affected.

Later work by Pitt and Barnes (1993) showed that massive novel protein phosphorylation occurred 2h after Ca^{2+} addition to *P. notatum.* The anti-calmodulin agent calmidazolium greatly reduced sporulation when addition of it preceded that of Ca^{2+}. Similarly, disturbance of Ca homeostasis was achieved with EGTA (ethlyene-bis(oxy-ethylenenitrolo) tetraacetic acid) and Ca channel blockers, resulting in the impairment of appressorium development of *Colletotrichum trifolii* during germination (Warwar and Dickman, 1996).

There was a clear difference in the levels of polyketide production on a biomass basis between shake and static cultures of *P. expansum* grown in ME medium. The ME shake cultures did not show great levels of polyketide production. Early pathway polyketides were favoured in the ME shake cultures up until the fourth day, when there was a general increase in the levels of these metabolites. The mycelium had increased in pigmentation at this time, presumably through the activity of diphenol oxidases, another indicator of a shift in metabolism towards secondary processes. This activity is related to polyketide biosynthesis because some of the hydroxy-phenol substrates for these enzymes are patulin pathway intermediates, and therefore the production of these would influence the substrates available for oxidative browning and pigmentation of the mycelium to occur.

The pigmentation of the ME mycelium compared to YE could reflect the greater significance of polyketide biosynthesis in the former due to the poorer nutritional

status of the medium, as well as differences in morphology, discussed earlier in this chapter. A more likely explanation is that a component of the ME medium induced the formation of diphenol oxidases. Fahraeus (1952) showed that malt extract produced good laccase activities in *Polyporus versicolor*, and Bocks (1967) showed the induction of the same activity in *A. bisporus*.

Secondary metabolism was favoured from the outset in the ME static cultures. Activation of secondary metabolism would have occurred as a result of the slow growth rate since oxidative metabolism would have low levels of terminal O_2 as well as the low NH_4^+ concentrations in this medium.

Toluquinol was produced at the start of these cultures, and it is possible that the DO levels at the surface of the early cultures might have been adequate for the MFO that produces this metabolite to be active, since there was only a fine dispersion of germinated mycelium at this time.

The ME static cultures increased in biomass at 48h, indicating a redirection of precursor flux to favour primary metabolism, since polyketide levels did not increase significantly at this time. Malic enzyme, NAD^+-ICDH (at very low levels) and $NADP^+$-ICDH were the only primary enzymes to increase activities during the static culture period. This would reflect a push of acetate towards energy generation for the organism, and coincides with an increase in growth rate. It would appear that the ME static cultures maintain a balance where the flux of precursors to secondary metabolism is the norm, and growth disrupts this balance. Patulin levels increased significantly on day 3 so that secondary metabolism was fully operational at this time. By the fourth day of the culture the rate of patulin biosynthesis decreased and the termination or repression of synthesis by patulin feeding back onto its biosynthetic pathway or intracellular proteinase activity as discussed later in Section 8.5 was probably occurring.

The TCA cycle represents a point at which pO_2 (or O_2 availability) can influence the flow of acetyl CoA into polyketide biosynthesis and this was demonstrated well in the experiments in which *P. expansum* was grown in ME medium. The effect is

substantiated in the link between the TCA cycle and the electron transport chain, in which O_2 is the terminal acceptor.

Agitation in the ME medium resulted in a number of effects contributing to a general draw of acetate away from polyketide biosynthesis. An increase in TCA activity was assumed from the increase in the activity of NAD-ICDH. As discussed in Section 8.6, the increase in TCA activity was accompanied by an increase in lipid accumulation, which presumably results from the activation of fatty acid biosynthesis through the accumulation of the TCA intermediate citrate. This activation of the fatty acid pathway could provide a significant influence on the flow of acetate to polyketide biosynthesis by functioning to draw/divert the flux of precursors away from it.

Note that NADPH would also influence the formation of lipid over polyketides, and this is also discussed in Section 8.6.

8.5 THE REGULATION OF SECONDARY METABOLISM IN *P. EXPANSUM.*

In the light of the above four mechanisms are considered to affect secondary metabolism according to experiments performed on *P. expansum.*

The effect of NH_4^+ has already been documented and discussed in Chapter 2 and 6 as a result of work by Rollins and Gaucher (1994). In this study neither of the media used were supplemented with NH_4^+ ions to determine any effects on polyketide production. However, NH_4^+ levels were determined throughout the culture period in both.

It is highly unlikely that NH_4^+ concentrations would be repressive to polyketide formation in the ME medium because the levels that were recorded in these studies were very low. It is possible that deamination of the medium could possibly lead to the formation of NH_4^+, however the nitrogen source for the medium, peptone, was at low levels and the release of NH_4^+ would produce localised areas of NH_4^+ ions. This

would not occur in the shake cultures, since agitation would produce homogeneity of the medium components.

The measured levels of NH_4^+ in the YE medium suggested that repression of polyketide synthesis should not occur. However the amino N analysis of the medium components indicated that there were substantial levels of this form of N in the medium. The rate of deamination potentially could have an effect on intracellular NH_4^+ levels, particularly during the initial rapid growth phase when medium breakdown would be at its greatest.

Therefore it is interesting to note that the patulin pathway was not significant over the first 3 days of the YE shake cultures, during which time rapid utilisation of the growth medium was occurring. It could be concluded that NH_4^+ was a significant regulatory influence during this time in this type of medium. The medium NH_4^+ level decreased rapidly to 48 hours in these cultures, and it would follow that there was a lag while protein synthesis occurred before patulin biosynthesis was detected after 72h.

By comparison NH_4^+ was released into the medium at 24h in the static YE cultures. The utilisation of medium components was much slower in the static cultures, so that an imbalance between deamination and NH_4^+ utilisation by the cells would be created leading to a small period of accumulation at this time. There was a big increase in the specific level of patulin production (i.e. patulin production per dry weight) at 72h in the YE static cultures, which correlated with a big decrease in the levels of NH_4^+ in the culture medium. This indicates a relationship between this parameter and patulin biosynthesis.

The activation of early and late parts of the patulin pathway at different times was also evident in the accumulation of the polyketide metabolites. It is likely that co-ordinate NH_4^+ repression occurs at more than one place in the pathway, at differing sensitivities for different genes. The earlier pathway enzymes would be derepressed at higher NH_4^+ concentrations than the later enzymes.

The feed of substrate into the patulin biosynthetic pathway is another significant regulatory mechanism. This mechanism appeared to have its greatest effect in the ME

medium and less so in the YE medium, probably because there was no ammonium repression occurring in the former to override this effect. Agitation has been shown to control the diversion of acetate to polyketide biosynthesis and has already been discussed in Section 8.4.

An increase in precursors to secondary metabolism was assumed to have occurred in ME 48h shake cultures when the activity of the TCA enzyme NAD^+-ICDH decreased and an increase in the production of patulin per weight of *P. expansum* occurred. The level of general metabolism in the ME static cultures was low throughout, and there were increases in both the TCA activity and patulin production over the 4 days of culture growth. This could be due to various pathways becoming active later due to the slower growth rate in static ME cultures. Increases in NAD^+-ICDH activity concomitant with increases in the biosynthesis of patulin could also reflect the metabolic isolation of differentiated regions of the fungal mycelium. Morphological differentiation tends to occur at the same time as biochemical differentiation, and represents a point at which those cells become metabolically isolated from the rest of the mycelium. Secondary metabolism is considered a component of the normal development of many fungi (Campbell, 1983). Penicilli and conidia of *Penicillium cyclopium Westling* contained pigment and the enzyme cyclopenase, involved in the synthesis of alkaloids (Nover and Luckner, 1974). Guzman-de-Pena and Ruiz-Herrera (1997) used the inhibitor diaminobutanone and non-sporulating mutants to show that aflatoxin biosynthesis and sporulation were linked in *A. parasiticus*. In the *P. expansum* cultures, the cells on the surface contacting the air in static cultures in which morphological differentiation has been initiated would also direct flux towards secondary metabolism. By comparison, the undifferentiated cells at the interface with the culture medium would be more likely to favour primary processes. Therefore it would be possible to have concurrent increases in both the TCA cycle and polyketide production in the bulk mycelium in the static ME cultures.

Metabolic isolation and differentiation was discussed in a theory proposed by Hansberg and Aguirre (1990; Chapter 1.1.2). Once a cell has reached a differentiated state or is part of a differentiated structure, it becomes isolated from the environment, and in particular O_2. This could check the TCA cycle and oxidative metabolism, therefore the flux of acetate through to polyketide biosynthesis would be favoured.

The switch in the flow of precursors from primary to secondary metabolism was less obvious in *P. expansum* grown in YE medium. NAD^+-ICDH activities peaked at 24h in the shake cultures and then decreased, while patulin levels increased slightly over the 4 day culture period. The static activities showed a clearer relationship. The static cultures also peaked at 24h then decreased, while patulin production per dry weight increased at a substantial rate from this time. This contradicts the observations made with the ME static cultures, however the difference could be explained by the better nutritional state of the organism in the YE medium. The onset of secondary metabolism would be less rapid in an environment where adequate nutrients are available. Once again, it should be noted that because these experiments were performed on bulk extracts of differentiated mycelium, specific parts of the mycelium could not be separated into regions of defined metabolic states, and therefore these results represent the overall state of metabolism for the whole mycelium.

A third means for controlling secondary metabolism that can be identified as a result of this work, is the concentration of NADPH, through the activity of the pentose phosphate pathway and to a lesser degree, malic enzyme activity. NADPH could not be measured in this work, and therefore assumptions are based on the activities of pathways that lead to its generation. A clear relationship could be demonstrated between increased pentose phosphate activity and decreased patulin levels. NADPH provides reducing power for biosynthetic processes, and its absence would tend to favour secondary pathways such as polyketide biosynthesis which would are not reductive.

Finally, mechanisms to terminate patulin biosynthesis appeared to play a role in these cultures since the rate of patulin biosynthesis was observed to level off or halt in older static cultures. Two mechanisms could be operating here. The first is that patulin could be inhibiting late pathway enzymes that result in its production in some sort of feedback mechanism. Patulin is able to interact with sulfhydryl groups on proteins to form adducts (Singh, 1967), which could inactivate the enzymes. Similarly, it could act at a binding site on the enzyme as an allosteric inhibitor, without necessarily causing irreversible inhibition of activity as the formation of adducts would.

The second termination mechanism, which has been shown to occur in *P. urticae* by Rollins and Gaucher (1995), is degradation of pathway enzyme activity by intracellular proteinase activity. The delay between pathway activation and degradation could easily be explained by the later activation/derepression of proteinase activity by ammonium ions also. The apparent termination of activity in the later portion of the patulin pathway and the continued accumulation of earlier pathway metabolites would appear to not be consistent with this notion however. Yet it may be possible that the later pathway enzymes are more susceptible through their conformation to the protease activity than the earlier ones, therefore explaining this apparent discrepancy.

8.6 LIPID ACCUMULATION.

Accumulation of lipid occurred to higher levels in the ME medium than in the YE medium, and to a greater extent in shake cultures compared to static cultures.

Initially, the higher amounts of lipid in the ME medium appear surprising given the limited nutritional status of this medium. However some hypotheses can be proposed to account for these results. The relationship of lipid accumulation to the growth phases outlined by Borrow *et al.* (1961, 1964) is discussed below (Section 8.7), and provides an insight into why there are greater levels of lipid accumulation in *P. expansum* under nutritionally poor conditions. Lipid accumulation occurs at the end of tropophase, during the transition phase to storage processes. The nutritionally poor ME medium would result in *P. expansum* moving through the balanced growth phase quickly so initiation of lipid and carbohydrate accumulation would have occurred. By contrast, growth on the YE medium would result in a significantly longer balanced growth phase through the availability of greater concentrations of essential nutrients to the organism compared to the ME medium, thus delaying the need for accumulation of carbon source for maintenance of cellular processes.

Catabolite repression of some primary processes is also likely to have occurred in the YE cultures due to the levels of intermediates that can be generated in a system grown in good nutritional conditions. The activities of the TCA enzyme, NAD^+-ICDH were

at similar levels for ME and YE cultures, despite the superior nutritional status of the YE medium. This suggests that this cycle was possibly under some kind of repression in the YE cultures. Assuming this to be the case, it may be assumed that this would prevent accumulation of TCA intermediates, citrate in particular, which is a signal for the initiation of fatty acid biosynthesis. Thus, the production of fatty acids and lipid accumulation would be less favoured in the YE medium compared to the ME medium.

The differences observed between shake and static can be accounted for in the relationship between the TCA cycle and fatty acid biosynthesis. The TCA cycle has a central relationship to several points in metabolism.

These include:

(i) transamination – the feed and bleed-off of TCA intermediates for amino acid metabolism;

(ii) energy generation through the coupling of the TCA cycle to the electron transport chain and oxidative phosphorylation;

(iii) The TCA is also strongly linked to O_2 levels because it is coupled to oxidative phosphorylation.

(iv) malic enzyme –whose role is still uncertain, but is a potential NADPH generator and is intimately linked to the TCA cycle;

(v) acetyl CoA metabolism through other pathways. As one of the primary routes for acetyl CoA utilisation, withdrawl or diversion to other acetate-utilising pathways such as fatty acid biosynthesis, polyketide biosynthesis has a large effect. The regulation of fatty acid biosynthesis by citrate, a TCA metabolite, is central to the relationship between this cycle and fatty acid synthesis.

The general increase in TCA activity of ME cultures due to the presence of O_2 through agitation was reflected in an increase in lipid accumulation. Presumably the increased accumulation of citrate under the more favourable shake conditions signalled the initiation of fatty acid production under these conditions. Lower TCA activity in the static cultures would have meant that there was not the same amount of TCA intermediate accumulation occurring, and therefore the synthesis of fat would not be initiated.

The static cultures grown in ME medium would be far more concerned with the maintenance of basic metabolism and energy generation. The ME medium would also present a situation in which the feed and bleed of TCA intermediates for amino acid metabolism and protein synthesis would have a significantly greater effect that the situation in the YE cultures, which had better nutrient availability.

Agitation had no effect on the TCA activities of YE cultures, and this was reflected in the lack of effect in lipid accumulation. Because there would have been significant levels of precursors in the YE cultures there would not have been a situation in which there would be bulk differences between the cultures that would have resulted in an observable difference in lipid accumulation. The specific activity of the lipid accumulation would have been affected by the diffusion of O_2 as discussed earlier in this Section 8.1, which was affected by the morphology of the organism. The lower diffusion of O_2 in the thick YE shake cultures would result in a reduced ability of metabolites to be pulled through the TCA cycle and therefore would appear to be no different in the dynamics of the TCA cycle and lipid accumulation to the static cultures.

A correlation can be drawn between the activities of the pentose phosphate cycle, the major provider of NADPH in this organism, and lipid accumulation, particularly in the ME medium. Although the shake and static activities were similar for both media for glucose-6-phosphate dehydrogenase, it was possible to show that an increase in the activity of this enzyme was concomitant with an increase in the accumulation of lipid in *P. expansum*. This suggests that the availability of reducing equivalents could direct the flux of acetyl CoA into fatty acid synthesis rather than polyketide synthesis. The same relationship was much harder to conclude in the YE cultures because of overlapping error margins between shake and static cultures.

The role $NADP^+$-ICDH in fatty acid synthesis should also be considered because of the proximity of this enzyme to fatty acid biosynthesis in the cell. Fatty acid biosynthesis is regulated without the control of this enzyme, however the activity of this enzyme would affect the flux of metabolites to it. The compartmentalised supply of reducing equivalents from this enzyme would affect the ability for reductive biosynthesis to occur. While the activities of $NADP^+$-ICDH were not high and

therefore not considered to make a significant contribution to the NADPH pool, the levels in shake cultures were elevated compared to static cultures in the ME medium. This correlated (or coincided) with an increase in the level of lipid accumulation.

The same relationships were not observed in the YE cultures. The levels of $NADP^+$-ICDH and lipid accumulations were in fact slightly decreased upon agitation despite a slight increase in the G6PD, despite the pentose phosphate pathway being expected to contribute more NADPH and therefore possibly elevate the lipid levels. A slight change in activity should be considered in the context of the bulk preparation of crude extracts. The activities obtained for these assays reflect the bulk of the mycelium and not localised portions of it, therefore a significant change in activity in a particular region would only manifest in a slight increase in the general activity if the rest of the mycelium was unaffected.

8.7 BORROW'S GROWTH PHASES AND *P. EXPANSUM*.

It is useful to consider the growth phases defined by Borrow *et al.* (1961, 1964) to help define where the parameters measured here fit into the metabolism of *P. expansum*. Borrow's phases are reviewed more fully in Chapter 2, however they can be described briefly as follows. The balanced growth phase describes growth that is able to occur without any nutrient limitations. This is followed by the transition phase in which growth is still occurring but one or two nutrients are now limiting (N and glucose have the greatest effect), and accumulation of mycelial fat/lipid and carbohydrate occurs. These two phases equate to Bu'Lock's Tropophase. Idiophase is marked by two phases – the storage phase, during which nutrients are no longer taken up from the medium and dry weight reaches its maximum; and the maintenance phase, when external and internal carbon is metabolised. Terminal phase is when autolysis of the mycelium occurs and ions are released back into the medium, with an increase in the medium pH as well.

Terminal phase was not recorded in any of these cultures but would have been observed if monitoring of the cultures had been conducted over a longer period of time.

Balanced growth would have occurred in all cultures over the first 24h, with the exception of the ME static cultures, in which polyketide synthesis was already initiated, and represented a period during which ammonium and reducing sugars were being taken up from the respective media.

The start of transition phase would best be determined in these cultures by a point at which lipid accumulation appeared to increase. This occurred at 48h in the ME shake cultures, and gradually from this time in the YE shake cultures. Lipid accumulation in the YE static cultures occurred to lower levels and earlier than the shake cultures, indicating an earlier onset of the transition phase. Lipid accumulation was not particularly significant in the ME static cultures, and indicates that this phase must have been earlier and short-lived in this situation.

Storage phase was reflected in parameters such as pH, the cessation of growth (dry weight), and of glucose and ammonium uptake, and decreased polygalacturonase activity. The decrease in the pH of both media was largely reflected in the increase in dry weight of the cultures.

This occurred at 72h in the ME and YE shake cultures (probably between 48-72h in the YE cultures, but this wasn't captured by measuring parameters at 24 h intervals), and after 96h in the static cultures. However, the timing of these do not necessarily coincide with the production of polyketides, which would be initiated during this period. In the ME static cultures this can be explained (as previously) by the very low ammonium levels in the culture medium. This would allow derepression of polyketide biosynthesis very early in the culture and the metabolic rate would be so low that there is no diversion of precursors to energy generation as for the shake cultures. This explanation does not hold for the YE static cultures, however differentiation of the mycelium in these cultures would seem a likely explanation.

The only evidence of maintenance phase in *P. expansum* that was observed here was the depletion of accumulated lipid in the YE static cultures, occurring at 48h. The ME static culture was presumably operating at such a low metabolic rate that lipid

accumulation was not favoured and acetate precursors tended to be directed to polyketide biosynthesis from early on anyway.

An inverse relationship between polyketide and fatty acid accumulation would show the depletion of lipid to feed patulin production was occurring in these cultures, particularly later in the culture period, when maintenance phase was reached. This relationship was evident in static cultures grown in YE medium. A decline in the accumulated lipid level coincided with an increase in the accumulated patulin level in the culture medium. This same decline was not evident in the shake cultures of both media, however polyketide production was not significant in these cultures for reasons outlined earlier in this discussion.

The shape of the curves for polyketide and fatty acid accumulation in the ME static cultures were similar (Figure 47, Chapter 5.5.4.2; Figure 55, Chapter 7). Fatty acid did not accumulate to a high level in these cultures anyway, presumably because of the slow metabolic rate.

A better tool to track the change in phases and metabolic turnover would be the use of probes to monitor the formation of specific indicator mRNAs at different times during the culture. This would give a better indication of the timing of the derepression/ induction processes. However, this would have expanded the workload in this thesis considerably.

8.8 CONCLUDING REMARKS.

The effect of agitation on the metabolism of *Penicillium expansum* can best be summed up by Figures 58 and 59, which demonstrate the effects observed in cultures grown in ME medium and YE medium respectively. Aeration clearly favoured the accumulation of lipid over polyketides by this organism in the ME medium through increased TCA cycle activity and NADPH generating pathway activities. The YE medium shake and static results were closer to each other, most probably because of the thick mycelial suspension that formed in shake cultures of this medium, and also

because the high carbohydrate concentration of this medium probably caused catabolite repression of some primary processes.

This thesis clearly demonstrates that agitation of fungal cultures affects the biosynthetic outcome. The switch between polyketide biosynthesis and lipid accumulation in this organism has been shown to originate in primary metabolism itself, through the accumulation of citrate from the TCA cycle and the availability of reducing power (NADPH) from the pentose phosphate shunt predominantly, with contributions likely from malic enzyme and cytoplasmic isocitrate dehydrogenase.

This work has also shown that inhibition of G6PD activity is effected strongly by not only its products (NADPH and 6-phosphogluconate), and erythrose from later in the pentose phosphate pathway, but also by ammonium, glutamine, AMP, ADP and ATP. Inhibition by ATP is reported in Levy (1979), who suggested also that the overall balance of adenine nucleotides in the cell could regulate the pentose phosphate shunt. These results strongly suggest that this enzyme is allosterically regulated by these metabolites. It is also likely that G6PD is activated allosterically by the binding of a positive effector under conditions favouring pentose phosphate activity. This project has not explored the identity of this effector, however it is possible it may be linked into the increased activity of the TCA cycle, through increased O_2 concentrations. It is feasible that increased electron transport chain activity could lead to increased levels of O radical species. These in turn could provide an intracellular signal to a phosphorylation cascade mediated by cAMP for example, or the phosphoinositol cascade discussed in Section 8.4. This would provide a universal signal in the switch from primary to secondary metabolism. This would be concordant with the hyperoxidant state of Toledo *et al.* (1995), in which an increase in hyperoxidant species is present at the start of each morphogenetic step in *N. crassa* conidiation.

This thesis has provided an overview of the workings of primary and secondary metabolism in *P. expansum*. Further work would need to focus on the enzyme activities involved in fatty acid synthesis since this study only quantified total neutral lipid accumulation by this organism, to give a clearer view of this aspect of metabolism. Likewise, it would be very interesting to determine the identity of positive effectors of pentose phosphate pathway activities so that the means by which

increased agitation translates into changes in the overall metabolic state can be determined.

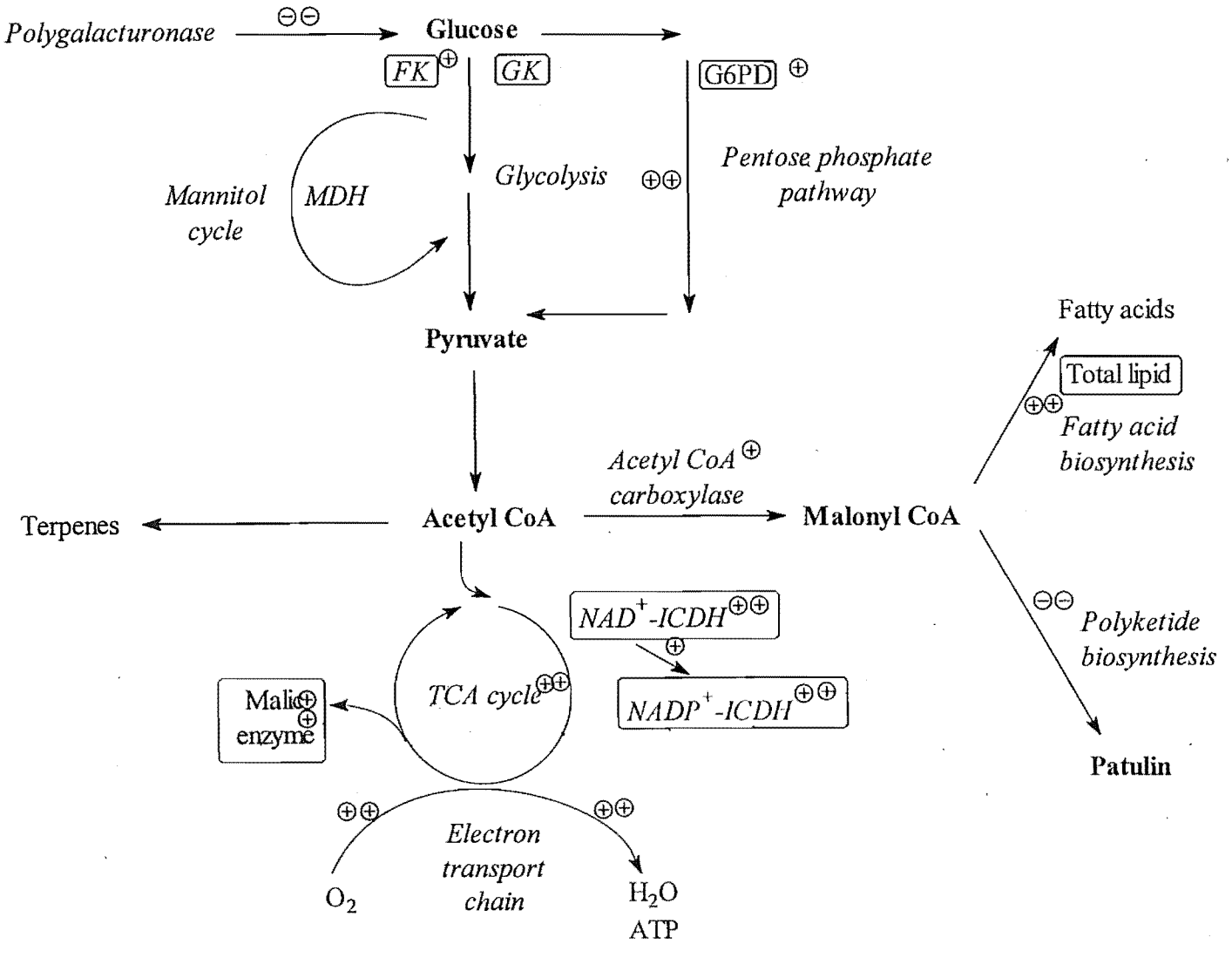


Figure 58. Overview of the effects of aeration on metabolism of *P. expansum* grown in Malt Extract medium.

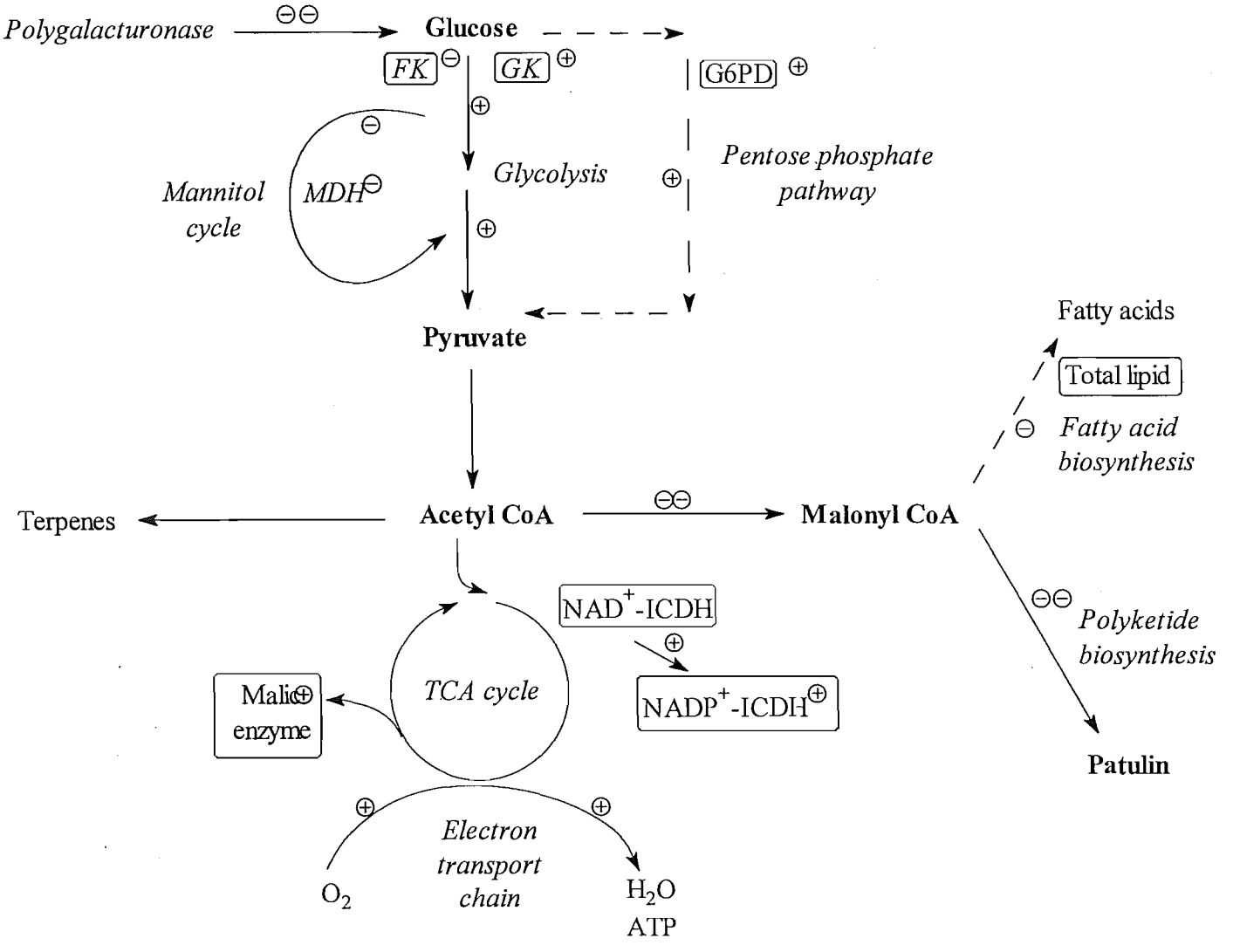


Figure 59. Overview of the effects of aeration on metabolism of *P. expansum* grown in YEGB medium.

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